Carnosine metabolism and function in the thoroughbred horse

Thesis

UNRESTRICTED

CARNOSINE METABOLISM AND FUNCTION IN THE THOROUGHBRED HORSE

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

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Declaration

I hereby certify that the work contained within this thesis has not already been accepted for any other degree, nor is it currently submitted in candidature for any other degree. Furthermore, I hereby certify that the work contained within this thesis is the result of my own investigation except where reference is made to published literature and where assistance is acknowledged.

(Candidate)

16th November 1995

Publications

This thesis is in part based on the following published manuscripts:

1. Determination of carnosine and other biogenic imidazoles in equine plasma by reversed-phase ion-pair high-performance liquid chromatography. M. Dunnett and R.C. Harris (1992) *J. Chromatogr.* 579 45-53.

2. Carnosine and taurine contents of type I, IIA and IIB fibres in the middle gluteal muscle. M. Dunnett and R.C. Harris (1995) *Equine vet. J., Suppl.* 18 214 - 217. (Proceedings of the Fourth International Conference on Equine Exercise Physiology).

3. High-performance liquid chromatographic determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in muscle and individual muscle fibres. M. Dunnett and R.C. Harris (1995) *J. Chromatogr. B.* (Accepted subject to revision and condensation).

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ABSTRACT

CARNOSINE METABOLISM AND FUNCTION IN THE THOROUGHBRED HORSE

Mark Dunnett

Thoroughbred horseracing involves high-intensity exercise characterized by the production and accumulation of hydrogen (H^+) ions within the skeletal muscles. Without a system for maintaining acid-base balance the consequential accumulation of H^+ ions within the working muscles would produce a rapid decline in intra-cellular pH with a concomitant impairment of the contractile process. Carnosine (ß-alanyl-L-histidine, pK_a 6.83) occurs at high concentration in equine muscle where it functions as an effective H^+ ion buffer at physiological pH.

High-performance liquid chromatography analytical methods were developed for carnosine and used to investigate its distribution and metabolism in equine fluids and tissues, with emphasis on type I, IIA and IIB muscle fibres. Foals and yearlings had significantly lower plasma carnosine concentrations than older horses. Plasma carnosine concentration showed little change during normal feeding and high-intensity exercise, however, episodes of equine exertional rhabdomyolysis produced large increases. Carnosine concentrations in tissues, such as the heart, liver and intestine were 10 to 100-fold lower than in skeletal muscle. Carnosine displayed a heterogeneous distribution within skeletal muscle. Its concentration in type IIA and IIB fibres was approximately 5-fold higher than in type I fibres.

Extensive, partly anaerobic training produced a 2-fold increase in the carnosine concentration in type IIA fibres, and an increase, although non-significant, in type I and IIB fibres. Thirty days of dietary ß-alanine and histidine supplementation produced an adaptive increase in ß-alanine and histidine bioavailability, and significant increases in the carnosine concentration in type IIA and IIB fibres.

A greater skeletal muscle carnosine concentration via training and/or ß-alanine and histidine supplementation would produce a corresponding increase in H^+ ion buffering capacity, which may reduce the rate of metabolic acidosis during high-intensity exercise, and possibly delay the subsequent onset of localized muscle fatigue.

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CHAPTER 1

GENERAL INTRODUCTION AND A REVIEW OF THE LITERATURE ON CARNOSINE

1.1 GENERAL INTRODUCTION

1.1.1 The historical and contemporary role of the horse in society

Of the many animal species domesticated during the evolution of human civilization the horse has probably made the greatest contribution to Man's social development. The versatility of this species in terms of strength, speed, endurance and amenability to training has resulted in its use in many areas of human endeavour such as, hunting, transport, agriculture, warfare and sport. The decline in the use of the horse as a working animal has been accompanied by a corresponding increase in equine sporting activities both commercially and for leisure purposes. Equine sporting activities are probably as old as the overall relationship between man and horse, and most likely originated from the practise of skills necessary for the survival of the individual and the community. The number of equine sports throughout the World is considerable and reflects the diversity of cultures in which the horse is found. Some equine sports include; flat-racing, steeplechasing (including hurdling and point-to-point racing), harness racing (including trotting and pacing), endurance racing, three-day eventing, show-jumping, dressage, carriage driving, fox and stag hunting, polo and vaulting. World-wide sporting activities are dominated both in terms of numbers of animals involved and numbers of events staged by track-based racing which mostly comprises flat-racing, steeplechasing and harness racing.

1.1.2 An outline of thoroughbred racing and breeding

The breeding and racing of thoroughbred horses is a vast commercial enterprise both nationally and internationally. Recent figures estimate that there are some 50,000 thoroughbred horses in the USA, and 3,500 in France. In Britain alone horse racing and breeding is a multi-billion pound industry. In the tax year 1993/94 the leviable betting turnover alone was estimated at £4,441 million (HBLB 1993/94). During the 1994 British racing season, including both flat and jump racing, there were 11,202 Thoroughbreds in training owned by 8,778 (monthly average) active owners from a registered total of 18,888. In addition there were 559 licensed trainers and a further 286 permit-holding trainers, 743 professional jockeys and 748 amateurs, and 5,908 other racing stable employees (Jockey-Club 1994). The majority of these horses contributed to a total

of 7,089 races in 1,102 fixtures at 60 racecourses resulting in 71,217 runners competing for total prize money of £54.8 million (BHB 1994). In 1994 there were 11,594 foals bred for the purposes of racing from 22,072 registered mares and 1,061 active stallions in Britain and Ireland (Jockey-Club 1994). Other major centres for Thoroughbred breeding and racing include Ireland, Australia, Japan, South Africa, Dubai and Hong Kong.

Thoroughbred flat-race horses are required to exercise over distances ranging from 5 - 20 furlongs (1000 - 4000 m) for approximately 1 - 5 min. Average speeds for a 5 furlong race can often reach 18 m s^{-1} and maximum speeds attained during racing over this distance can approach 22 m s^{-1}. Thoroughbred steeplechasers race over longer distances of 16 - 36 furlongs (3200 - 7200 m). Although average speeds are lower the horses are required to negotiate between 6 - 9 hurdles or fences per mile. Both forms of racing provide a severe test of horses strength, speed and stamina.

1.1.3 Metabolic acidosis and intra-cellular hydrogen ion buffering during exercise

Production and accumulation of large quantities of lactic acid, and lesser amounts of pyruvic, malic and other acids, occurs in the working skeletal muscles during periods of high-intensity exercise as a consequence anaerobic glycolysis. At physiological pH carboxylic acids dissociate into carboxylate anions (i.e. lactate) and hydrogen (H^+) ions. It has been reported that during fatiguing exercise that lactic acid production is responsible for 94% of the total H^+ ion load in human skeletal muscle (Hultman and Sahlin 1980). Lactate concentrations in the equine middle gluteal muscle in excess of 200 mmol kg^{-1} dry weight (DW) following both racing (Valberg 1987) and repeated bouts of maximal treadmill exercise (Snow *et al.* 1985) have been observed. However, following both single and repeated bouts of maximal exercise, lactate concentrations in the middle gluteal muscle of the horse of 150 mmol kg^{-1} DW are more common (Harris *et al.* 1989; Harris *et al.* 1987). Such a muscle lactate concentration is representative of a H^+ ion production of 150 000 μmol kg^{-1} DW which would theoretically cause a fall in the intra-cellular pH to a value of 1.0 - 2.0. In practice however, a reduction in intra-cellular pH from a resting value of 7.1 to a post-exercise value of 6.5 represents an increase in H^+ ion concentration of only

0.71 μmol kg^{-1} DW (Marlin and Harris 1991). Although a proportion of the H^+ ions produced will be exported from the skeletal muscle to the blood there remains a strong correlation (r = 0.97) between muscle pH and the lactate and pyruvate concentration (Harris *et al.* 1989), and hence the majority of the H^+ ions produced can be accounted for through buffering within the muscle.

Mammalian skeletal muscle is reliant upon several different buffering mechanisms which operate in concert to regulate intra-cellular pH homeostasis. Overall or total intra-cellular H^+ ion buffering (βm_{total}) in skeletal muscle can be considered to have three major components: physico-chemical buffering, metabolic buffering and dynamic buffering. The physico-chemical component can be further divided into two components. First, intrinsic buffers which include; the weak acid-base moieties associated with proteins, the histidine and 3-methylhistidine residues of actin and myosin, the imidazole dipeptides carnosine, anserine and balenine, and free histidine. Second, extrinsic buffers, such as bicarbonate. The metabolic buffering component comprises the dephosphorylation of ATP to IMP, NH_4^+ and inorganic phosphate, dephosphorylation of phosphocreatine, and NH_4^+ ion production during amino acid catabolism. The dynamic buffering component regulates the intra-cellular H^+ ion concentration via transmembrane H^+ ion flux, for example from the cytosol to the blood plasma.

The imidazole dipeptides can account for a significant proportion of the intra-cellular physico-chemical buffering (Abe *et al.* 1985), although the exact proportion is highly variable between species (Okuma and Abe 1992).

1.2 REVIEW OF THE LITERATURE ON CARNOSINE

1.2.1 Discovery and chemical structures of carnosine and related compounds

The imidazole dipeptide carnosine (ß-alanyl-L-histidine) is a polar, water soluble, small molecular weight compound (MW 226.2) which exists in the zwitterionic form at pH 7.0 (Figure 1.1). The molecule possesses three ionizable groups; a terminal amino group (pK_a 9.66), a carboxylate group (pK_a 2.77) and an imidazole ring (pK_a 6.83) (Tanokura *et al.* 1976). Carnosine also possesses a chiral carbon atom and hence can exist as both the L- or the D-isomer. The L-isomer is the naturally occurring form of the compound.

Carnosine (ß-alanyl-L-histidine) was first isolated from Liebig's meat extract (Gulewitsch and Amiradzhibi 1900a; Gulewitsch and Amiradzhibi 1900b). Five years later, a German biochemist isolated a nitrogenous organic compound from the same source which was named ignotine (Kutscher 1905). Subsequent comparative analysis established that carnosine and ignotine were identical (Gulewitsch 1906/7). Gulewitsch established the dipeptide nature of carnosine by demonstrating that it was hydrolysed to histidine (Gulewitsch 1906/7) and ß-alanine (Gulewitsch 1911). The structure of carnosine was confirmed following its chemical synthesis (Barger and Tutin 1918; Baumen and Ingvaldsen 1918). Naturally occurring methylated analogues of carnosine were soon discovered. Anserine (ß-alanyl-L-1-methylhistidine) was first isolated from skeletal muscle of the chicken and goose (Ackermann *et al.* 1929; Tolkachevskaya 1929) and its structure was confirmed following its chemical synthesis (Behrens and du-Vigneaud 1937). Balenine, also known as ophidine (ß-alanyl-L-3-methylhistidine), was discovered in skeletal muscle of the Cobra (Imamura 1934), however, for many years it was thought to be ß-alanyl-L-2-methylhistidine. The correct chemical structure for this dipeptide was later established using nuclear magnetic resonance spectroscopy (Wolff *et al.* 1968). Homocarnosine (γ-aminobutyryl-L-histidine), a dipeptide analogue of carnosine in which the ß-alanine residue is replaced by γ-aminobutyric acid , was discovered in bovine brain (Pisano *et al.* 1961). N-α-acetyl-L-carnosine, a carnosine derivative in which one of the hydrogen atoms of the terminal amine group replaced by an acetyl group, was discovered in the central nervous system (CNS) and skeletal muscle of

Figure 1.1 Empirical formula and chemical structure of carnosine. (* Denotes the chiral centre).

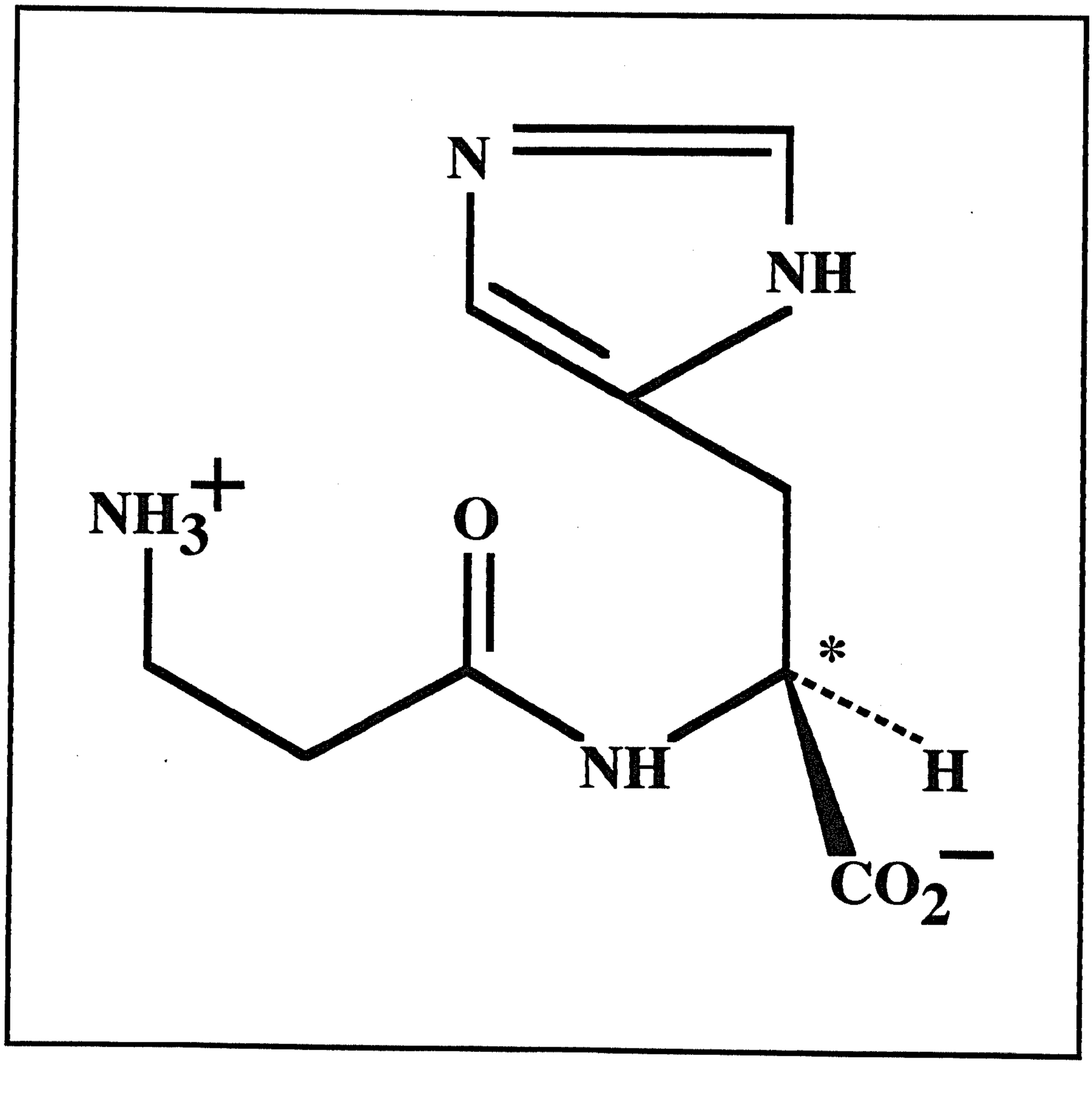

N
NH
NH3+
O
NH
*
H
CO2−

Table 1.1 Discovery of carnosine and related compounds. Adapted from Boldyrev and Severin (1990)

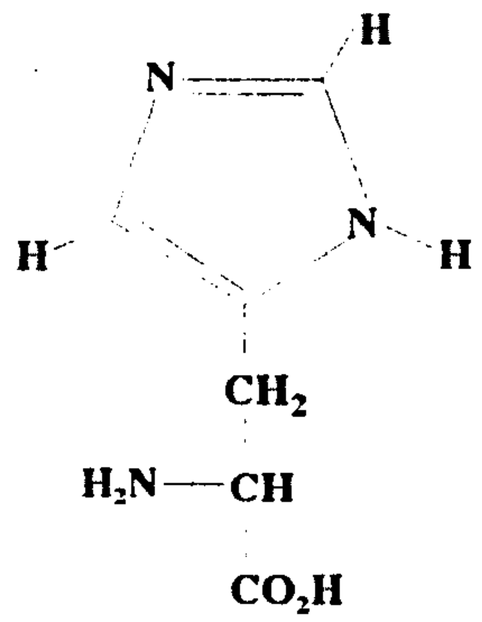
Common Name	Name	Source	Reference
Carnosine	ß-alanyl-L-histidine	Meat extract	Gulewitsch and Amiradzhibi (1900)
Anserine	ß-alanyl-L-1-methylhistidine	Chicken muscle	Tolkachevskaya (1929)
		Goose muscle	Ackermann *et al.* (1929)
Balenine	ß-alanyl-L-3-methylhistidine	Cobra muscle	Imamura (1934)
Homocarnosine	γ-aminobutyryl-L-histidine	Human CNS	Pisano *et al.* (1961)
Homoanserine	γ-aminobutyryl-L-1-methylhistidine	Bovine CNS	Nakajima *et al.* (1969)
Acetylcarnosine	N-α-acetyl-ß-alanyl-L-histidine	CNS	Sobue *et al.* (1975)
Acetylhomocarnosine	N-α-acetyl-γ-aminobutyryl-L-histidine	CNS	Sobue *et al.* (1975)
Acetylanserine	N-α-acetyl-ß-alanyl-L-1-methylhistidine	Myocardium	O'Dowd *et al.* (1988)
Carcinine	ß-alanylhistamine	Crab CNS	Arnould and Frentz (1975a)

Figure 1.2 Empirical formulae, chemical structures and molecular weights of histidine and other imidazole dipeptides.

HISTIDINE

$C_6H_9N_3O_2$

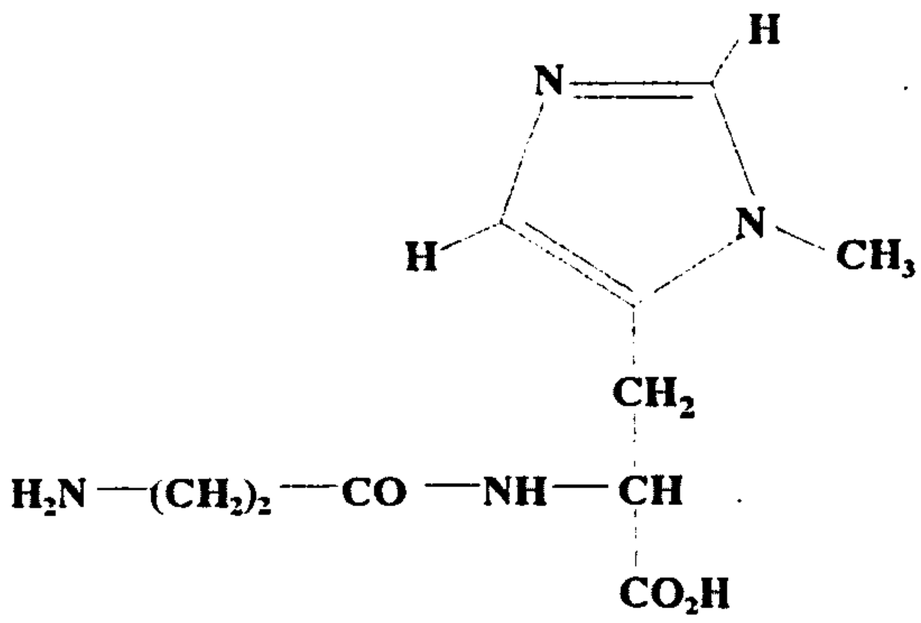
MW 155.2



ANSERINE

$C_{10}H_{16}N_4O_3$

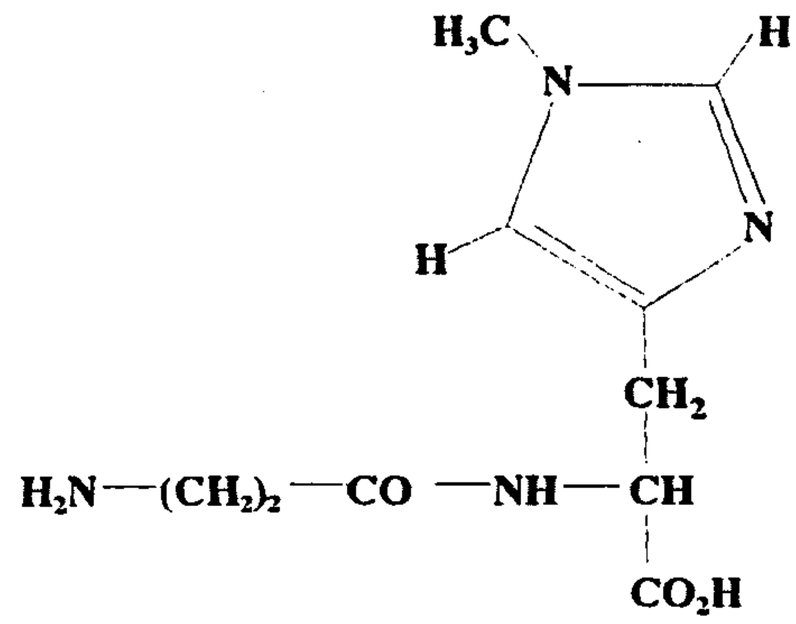
MW 240.2



BALENINE

$C_{10}H_{16}N_4O_3$

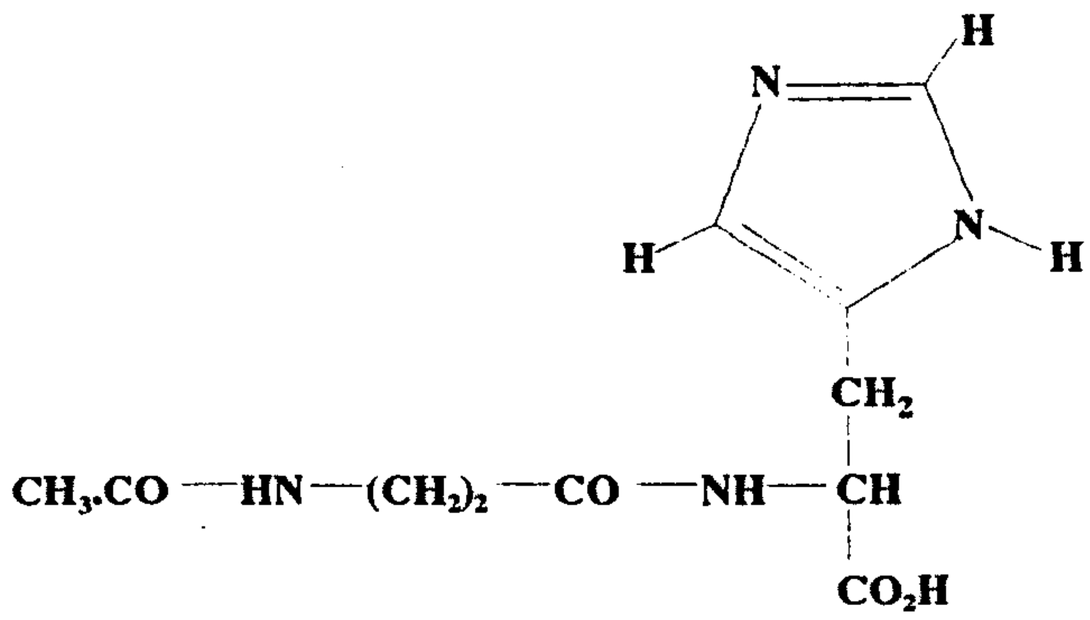
MW 240.2



N-ACETYLCARNOSINE

$C_{11}H_{16}N_4O_4$

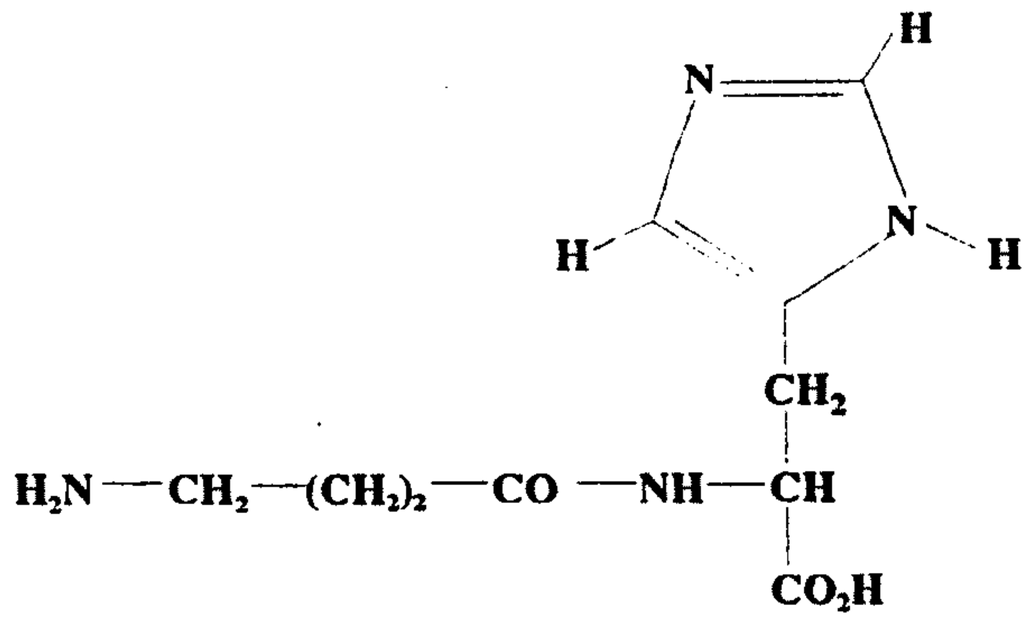
MW 268.2



HOMOCARNOSINE

$C_{10}H_{16}N_4O_3$

MW 240.3



the frog (Sobue *et al.* 1975). These and other carnosine related imidazole dipeptides which have since been discovered are listed in Table 1.1. The empirical formulae, chemical structures and MW of histidine and some imidazole dipeptides are shown in Figure 1.2.

1.2.2 Occurrence and distribution of carnosine and related compounds in nature

Carnosine distribution in non-vertebrate species

Carnosine and related compounds have a wide though heterogeneous phylogenetic distribution. To date the occurrence of the imidazole dipeptides has been investigated in more than 80 species. The literature appears to contain no reports of the presence of carnosine in the tissues of either plants or micro-organisms, although histidine is present in both plant and animal proteins. ß-alanine is reported to be present at low concentrations in some plants, such as green coffee beans (2.7 - 5.7 mg kg^{-1} DW or 0.03 - 0.06 mmol kg^{-1} DW) (Arnold *et al.* 1994) and the cycad *Stangeria eriopus* (not quantified) (Osborne *et al.* 1994). Green coffee beans also contain free 1-methylhistidine (< 50 mg kg^{-1} DW or 0.3 mmol kg^{-1} DW), 3-methylhistidine (25.0 - 43.2 mg kg^{-1} DW or 0.16 - 0.28 mmol kg^{-1} DW) and γ-aminobutyric acid (not quantified) (Arnold *et al.* 1994). Carnosine is found in some arthropods such as, the lavae of the blowfly (not quantified) (Bodnaryk and Levenbrook 1968). Carnosine (and anserine) have been detected in some species of molluscs and crustaceans (Luckton and Olcott 1958), although they are reported to be absent in several others (Suyama *et al.* 1970). Some species of molluscs and crustaceans and many fish, however, appear to possess much greater amounts of the amino acid precursors of dipeptides, histidine and ß-alanine, rather than the dipeptides themselves (Luckton and Olcott 1958). More recently a previously unknown imidazole dipeptide, carcinine (ß-alanylhistamine), was detected in the CNS and muscle of the crustacean *Carcinus maenas* (Arnould and Frentz 1975a; Arnould and Frentz 1975b) and later in other crustacea (Arnould 1986). The typical concentrations of the imidazole dipeptides in the tissues of these species are shown in Table 1.2.

Table 1.2 Mean concentrations of carnosine and related compounds in the muscle tissue of invertebrates.

Species	Concentration (mmol kg^{-1} DW)			Reference
	Carnosine	Anserine	Balenine	
Blowfly (lavae)	+	ND	ND	Bodnaryk and Levenbrook (1968)
Crab	0.5	0.5	ND	Suyama *et al.* (1970) †
Crab	13.3	ND	-	Cameron (1989)
Crab	0.8	ND	-	Luckton and Olcott (1958)
Squid	2.0	ND	-	Luckton and Olcott (1958)
Shrimp	8.0	ND	-	Luckton and Olcott (1958)
Scallop	1.2	ND	-	Luckton and Olcott (1958)
Abalone	2.0	ND	-	Luckton and Olcott (1958)
Oyster	0.8	6.4	-	Luckton and Olcott (1958)

† = Carnosine, anserine and balenine were not detected in the muscle tissue of shrimp, hard clam, gaper, top-shall, abalone, squid, octopus and sea cucumber.

\+ = Detected but not quantified.

ND = Not detected.

\- = Not determined.

Carnosine distribution in tissues

Skeletal muscles of vertebrates contain very high concentrations of carnosine and other imidazole dipeptides, often in excess of 50 mmol kg^{-1} DW. Early investigations by (Wood 1957) and (Davey 1960a), using colourimetric and ion-exchange assays, tentatively suggested the presence of carnosine in rat stomach but did not detect the peptide in other tissues, including heart, kidney and liver, nor in the heart of horses. Subsequent improvements in analytical techniques have shown that low millimolar concentrations of carnosine and the other imidazole dipeptides occur principally in heart and tissues of the CNS and gastro-intestinal tract (GIT) of several species including the rat, rabbit, guinea pig, frog, mouse, pigeon, chick and man. However, the distribution between different tissues of a given species and between species is highly variable.

Sobue *et al.* (1975) reported carnosine concentrations in rabbit heart, liver and kidney of 618, 441 and 39 nmol g^{-1} WW (2.47, 1.76 and 0.16 mmol kg^{-1} DW), respectively. Similar values in cardiac muscle were reported by O'Dowd *et al.* (1988) in rat, guinea pig and frog. The retina of pigeons and chickens contain 0.08 - 0.26 μmol g^{-1} WW (0.32 - 1.04 mmol kg^{-1} DW) of carnosine and anserine concentrations of 1.4 - 5.2 μmol g^{-1} WW (5.6 - 20.9 mmol kg^{-1} DW) (Margolis and Grillo 1984). In contrast, carnosine was almost invariably absent from mammalian (including human) retina, however, homocarnosine was present at concentrations of 0.05 - 0.10 μmol g^{-1} WW (0.2 - 0.4 mmol kg^{-1} DW). Retinal carnosine concentrations were highest in the frog (6.4 mmol kg^{-1} DW). Both carnosine and anserine were found at low concentrations in other regions of the avian CNS including chicken cerebellum, cerebral hemispheres and optic lobes (Margolis and Grillo 1984). Within the CNS of vertebrates studied, including the hamster, pig, dog, rabbit and rat, the highest concentrations of carnosine are localized within the olfactory bulb and epithelium (Ferriero and Margolis 1975; Neidle and Kandera 1974). The carnosine concentration in mouse olfactory bulb was 0.9 - 2.2 nmol mg^{-1} WW (3.6 - 8.8 mmol kg^{-1} DW) (Ferriero and Margolis 1975; Neidle and Kandera 1974; Wideman *et al.* 1978), and in the Geko a concentration of up to 3.9 μmol g^{-1} WW (15.6 mmol kg^{-1} DW) was reported (Margolis 1981). In other regions of the mammalian CNS, such as the cerebellum, cerebrum and brain stem,

homocarnosine, with tissue concentrations up to 52.4 μmol 100 g^{-1} WW (2.1 mmol kg^{-1} DW), is the major imidazole dipeptide present (Abraham *et al.* 1962). In human brain homocarnosine concentration was 2 to 4-fold higher in white matter than in grey matter (Abraham *et al.* 1962).

Flancbaum *et al.* (1990) reported the most comprehensive study of carnosine concentrations in tissues other than skeletal muscle when investigating a possible relationship between carnosine and histamine concentrations in the rat, mouse, guinea pig and man (see section 1.2.4 *Histamine synthesis in response to shock and wound healing*). These mean values of carnosine concentrations and those determined by other investigators are given in Table 1.3.

The total concentration of the acetylated forms of the imidazole dipeptides, N-α-acetylcarnosine, N-α-acetylanserine and N-α-acetylhomocarnosine, in cardiac muscle and tissues of the CNS (other than olfactory bulb) exceeds the concentrations of the non-acetylated forms. The estimated total concentration of the acetylated imidazole dipeptides in cardiac muscle is greater than 10 mmol kg^{-1} WW (40 mmol kg^{-1} DW) (O'Dowd *et al.* 1988).

Table 1.3 Mean carnosine concentrations in tissues of the rat, mouse, guinea pig and man.

Adapted from Flancbaum *et al.* (1990).

Tissue	Mean ± SD carnosine concentration (mmol kg^{-1} DW)			
	Rat	Mouse	Guinea pig	Man
Heart	0.44 ± 0.06	0.19 ± 0.06	0.31 ± 0.07	0.19 ± 0.02
Kidney	0.29 ± 0.05	0.43 ± 0.09	0.16 ± 0.07	1.08 †
Stomach	0.19 ± 0.03	0.24 ± 0.14	0.08 ± 0.02	0.22 ± 0.10
Liver	0.19 ± 0.03	0.16 ± 0.08	0.15 ± 0.04	-
Spleen	0.14 ± 0.03	0.25 ± 0.07	0.14 ± 0.04	-
Lung	0.21 ± 0.10	0.39 ± 0.13	0.06 ± 0.03	-
Ileum	0.11 ± 0.03	0.27 ± 0.06	0.15 ± 0.06	-
Hypothalamus	0.07 ± 0.01	0.46 ± 0.07	0.05 ± 0.02	-
Pituitary	0.25 ± 0.06	-	0.03 ± 0.02	-
Olfactory bulb	0.20 ± 0.09	0.55 ± 0.09	0.09 ± 0.04	-
Jejunum	-	-	-	0.31 ± 0.10

† = Only one sample.

Table adapted from Flancbaum *et al.* (1990)

Carnosine in vertebrate skeletal muscle

The concentrations of imidazole dipeptides found in vertebrate skeletal muscle are highly variable between species (Carnegie *et al.* 1983; Crush 1970; Plowman and Close 1988). Skeletal muscle of the Little Piked Whale contains the highest reported concentration of imidazole dipeptides with a total concentration of 2153 mg % or approximately 380 mmol kg^{-1} DW of which balenine contributed 350 mmol kg^{-1} DW (Suyama *et al.* 1970). Either carnosine, anserine or balenine, or a combination of these, occur at high concentrations in the skeletal muscle of almost all of the vertebrate species studied. The particular dipeptide, or combination of dipeptides present and their intra-muscular concentrations are extremely variable. From the very large number of species studied to date it is apparent that great similarities exist between the dipeptide ratios in individual species within the same zoological class. In general anserine predominates over carnosine in birds, balenine predominates over carnosine in reptiles and aquatic mammals whereas greater amounts of carnosine than anserine occur in many land based mammals. Balenine is absent from almost all land mammals (Crush 1970). The carnosine : anserine : balenine ratios in the skeletal muscles of different species have been studied in an attempt to establish a simple diagnostic test for the identification of the composition of consumer meat products (Carnegie *et al.* 1983; Plowman and Close 1988). Some examples of the concentrations of carnosine and related compounds in the skeletal muscles of different species are given in Table 1.4.

The incidence of a single dipeptide occurring in isolation in the skeletal muscle of a given species is comparatively rare. Carnosine alone appears to be present in only humans (Christman 1976) and equine skeletal muscle (Bump *et al.* 1989; Davey 1960a; Harris *et al.* 1990; Marlin *et al.* 1989). Christman (1976) reported carnosine concentrations in human psoas muscle in the range 1.0 - 7.7 μmol g^{-1} WW (4.0 - 30.8 mmol kg^{-1} DW). Anserine was not detected. These results have been confirmed by several subsequent investigations (Harris *et al.* 1990; Mannion *et al.* 1992; Parkhouse *et al.* 1985). The carnosine and anserine concentrations in human skeletal muscle in contrast to values from two other competitive athletic species, the greyhound and camel, are given in Table 1.5.

Table 1.4 Mean concentrations of carnosine and related compounds in the skeletal muscle of vertebrates.

Class	Species	Muscle	Mean ± SD concentration (mmol kg^{-1} DW)			Reference
			Carnosine	Anserine	Balenine	
Aves	Chicken	Pectoralis	70.7 ± 19.8	136.8 ± 3.6	ND	Plowman and Close (1988)
	Pigeon	Pectoralis	1.8†	23.3†	ND	Crush (1970)
	Goose	Pectoralis	16.7 ± 8.1	108.3 ± 16.1	-	Wolos *et al.* (1983)
	Rook	NS	-	58.3†	-	Boldyrev and Severin (1990)
Amphibia	Toad	Sartorius	31.8†	-	-	Briner (1961)
	Frog					
Reptilia	Sea snake	-	-	-	93.2†	Tsunoo *et al.* (1964)
	King cobra	-	ND	ND	20.0†	Crush (1970)
Osteichthyes	Tuna	-	1.7†	181.5†	1.1†	Suyama *et al.* (1970)
	Salmon	-	ND	70.7†	ND	Crush (1970)
Mammalia	Zebra	Gluteus medius	112.4 ± 11.3	ND	ND	Dunnett, unpublished)
	Donkey	NS	48.4 ± 24.0	0.4 ± 0.1	ND	Carnegie *et al.* (1983)
	Kangaroo	NS	9.2 ± 7.6	63.6 ± 15.2	ND	Carnegie *et al.* (1983)
	Wallaby	NS	9.6†	123.6†	ND	Plowman and Close (1988)
	Hare	NS	20.0 ± 7.2	93.0 ± 10.8	ND	Plowman and Close (1988)
	Rabbit	NS	15.4 ± 7.7	77.6 ± 13.9	ND	Plowman and Close (1988)
	Pig	NS	48.4 ± 19.6	2.6 ± 0.3	3.0 ± 1.5	Carnegie *et al.* (1983)
	Deer	NS	34.2 ± 8.8	39.1 ± 11.0	11.3 ± 4.5	Plowman and Close (1988)
	Dolphin	NS	79.1†	15.4†	81.5†	Suyama *et al.* (1970)
	Whale	NS	40.1†	6.5	85.8†	Suyama *et al.* (1970)

ND = Not detected. NS = Not specified. † = Single sample. - = Not determined.

Table 1.5 Mean muscle carnosine and anserine concentrations in the skeletal muscles of man, greyhound and camel.

Species	Muscle	Concentration (mmol kg^{-1} DW)		Reference
		Carnosine	Anserine	
Man	Vastus lateralis	16.0 ± 7.2	ND	Harris *et al.* (1990)
	Quadriceps femoris	20.0 ± 4.7	ND	Mannion *et al.* (1992)
	Quadriceps femoris	21.8 ± 4.1	ND	Mannion *et al.* (1995)
Greyhound	Various muscles‡	33.0 ± 19.1	48.6 ± 18.4	Harris *et al.* (1990)
Camel	Gluteus medius	28.7 ± 14.9	36.9 ± 12.4	Dunnett and Harris (1995c)

ND = Not detected.

‡ = semitendonosis, biceps femoris, triceps, deltoid and longissimus dorsii.

The horse has been shown to possess one of the highest skeletal muscle imidazole dipeptide concentrations recorded amongst land based species. Early measurements of the carnosine concentration in equine skeletal muscle by Zapp and Wilson (1938) reported carnosine concentrations of 463, 436 and 161 mg/100 g WW (approximately 81.9, 77.1 and 28.5 mmol kg^{-1} DW) in the gluteus medius, gastrocnemius and soleus muscles, respectively. Anserine concentrations of 17 - 48 mg/100g WW (2.8 - 8.0 mmol kg^{-1} DW) were also reported, however this later finding has not been supported by subsequent work. A carnosine concentration of 25.5 μmol g^{-1} WW (102.0 mmol kg^{-1} DW) was detected in equine longissimus dorsi muscle (Davey 1960a). Slightly higher concentrations of between 108 and 134 mmol kg^{-1} DW have been reported more recently by several investigators (Bump *et al.* 1990; Harris *et al.* 1990; Marlin *et al.* 1989; Miller-Graber *et al.* 1990; Sewell *et al.* 1992). Furthermore, some differences in the skeletal muscle carnosine concentrations between various breeds of horses has been indicated. American Quarterhorses were shown to have higher concentrations than thoroughbred horses, which in turn had higher concentrations than standardbred horses (Bump *et al.* 1989; Bump *et al.* 1990). Carnosine concentrations in the middle gluteal muscle of Quarterhorses, Thoroughbreds and Standardbreds are given in Table 1.6.

Sub-cellular distribution of carnosine in skeletal muscle

It probable that carnosine exists predominantly in the free state in equine skeletal muscle with less than 10% present in a bound form (Bock 1958). A subsequent investigation which compared the dialysis rates of carnosine from aqueous standard solutions and fresh homogenates of frog skeletal muscle concurred with the earlier findings in equine muscle (Bock and Langley 1960). Evidence from the analysis of fractionated homogenates of rat gastrocnemius muscle indicates that 99.5% of the total imidazole dipeptide content is contained within the 15000g supernatant phase with only 0.3% present in the washed mitochondria and no detectable amounts of imidazole dipeptides in the residue. This suggests that the imidazole dipeptides are cytosolic in origin. Following intra-peritoneal administration of histidine-C^{14} or ß-alanine-C^{14} to chicks and subsequent isolation of radio-labelled imidazole dipeptides, it was found that 99% of the radioactivity was present in the supernatant, 1% of the radioactivity was in the myofibrils and

Table 1.6 Mean muscle carnosine concentrations in different breeds of horses.

Breed	Muscle	Carnosine mmol kg^{-1} DW	Reference
Thoroughbred	Gluteus medius	108.6 ± 15.2	Marlin *et al.* (1989)
	Gluteus medius	108.3 ± 15.9	Harris *et al.* (1990)
	Gluteus medius	125.3 ± 11.6	Bump *et al.* (1990)
	Gluteus medius	133.6 ± 20.7	Miller-Graber *et al.* (1990)
	Gluteus medius	125.0 ± 26.0	Sewell *et al.* (1992)
	Gluteus medius	107.8 ± 12.9	Dunnett and Harris (1995b)
Standardbred	Gluteus medius	110.6 ± 2.4	Bump *et al.* (1990)
Quarterhorse	Gluteus medius	156.8 ± 7.1	Bump *et al.* (1990)
	Glutius medius	159.1 ± 11.8	Bump *et al.* (1989)

less than 0.1% was present in mitochondria and microsomes (Winnick *et al.* 1963). Similarly, Harding and O'Fallon (1979) have reported that approximately 70% of the carnosine found in mouse olfactory bulb and epithelium was localized within the cytosol.

Effect of training and exercise on muscle carnosine content

Seven weeks of sub-maximal training resulted in a small but non-significant decrease in the carnosine concentration in equine middle gluteal muscle (Bump *et al.* 1989). Mean muscle carnosine concentrations, as described earlier, are higher in human subjects trained for high-intensity exercise such as sprinting and rowing in contrast to untrained individuals or those trained for sustained exercise of lower intensity, such as marathon running (Parkhouse *et al.* 1985). However, the higher muscle carnosine values evident in sprinters and rowers may well arise from a genetic predisposition rather than a training effect.

Bump *et al.* (1989) reported a significant reduction ($p < 0.05$) in the carnosine concentration in equine middle gluteal muscle from a pre-exercise value of approximately 140 mmol kg^{-1} DW to a value of 112 mmol kg^{-1} DW immediately following a sub-maximal exercise bout which resulted in a mean peak blood lactate concentration of 9.7 m*M*. However, this finding was refuted in a later study by Harris *et al.* (1992) in which the carnosine concentration in the middle gluteal muscle of the thoroughbred horse was unchanged from the pre-exercise value immediately following 4 x 700 m bouts of near maximal exercise which resulted in a mean peak blood lactate concentration of 26.8 m*M*. The changes previously reported by Bump *et al.* (1989) probably arose as a result of the use of wet tissue and could be attributed to an exercise induced increase in the blood content of muscle samples (Harris *et al.* 1992). No significant change in the muscle carnosine concentration of the gluteus medius of thoroughbred horses was found during fatiguing exercise (Miller-Graber *et al.* 1990).

1.2.3 Skeletal muscle carnosine and hydrogen ion buffering

Carnosine is an organic base. The imino nitrogen atom of the imidazole ring of carnosine and anserine have pK_a values of 6.83 and 7.04, respectively, and are capable of accepting (binding)

H^+ ions (Tanokura *et al.* 1976). Compounds with a capacity for buffering H^+ ions function most effectively when the pH of the environment is within one pH unit of the pK_a value. Carnosine will buffer most effectively between pH 5.83 and 7.83 and is therefore able to function as an efficient H^+ ion buffer over the physiological pH range. The H^+ ion buffering role of carnosine (and anserine) in skeletal muscle was first proposed as a significant component of the total intracellular physico-chemical muscle buffering capacity (ß*m*) by Bate-Smith (1938). This hypothesis was supported by the discovery that the pK_a values for carnosine and anserine were approximately 6.9 and 7.1, respectively (Bate-Smith 1938; Deutsch and Eggleton 1938). It was calculated that carnosine accounted for 20 - 25% of the post-rigour buffering in equine muscle between pH 5.5 and 7.5. Furthermore, it was estimated that it may have contributed approximately 40% of the total in pre-rigour muscle. Davey (1960a) reached a similar conclusion that carnosine and anserine accounted for the major proportion (up to 40%) of the total buffering of extracts of pre- and post-rigour muscle in several species between pH 7.5 and 6.5 . The hypothesis that carnosine functioned as a H^+ buffer *in vivo* was strengthened by the work of Severin *et al.* (1963) which demonstrated that electrically stimulated frog sartorius muscle immersed in Ringer's solution supplemented with carnosine or anserine (4 - 12 m*M*), in contrast to an almost identical muscle immersed in Ringer's solution only, showed a greater amplitude of contraction and a longer time to fatigue. In addition the accumulation of lactate was reportedly lower in the carnosine or anserine perfused muscle cited in (Severin *et al.* 1963). Whitaker and Louw (1984) reported similar findings when frog gastrocnemius muscle '*in situ*' was electrically stimulated via the ipsilateral sciatic nerve (proximal blood and nerve supply was intact) whilst perfused with either Ringer's solution or Ringer's including 20 m*M* carnosine or histidine. In the presence of histidine or carnosine, the time to exhaustion was significantly longer ($p < 0.01$), and lactate accumulation and K^+ ion efflux from the muscle were reduced but not significantly. They concluded that fatigue was reduced in the presence of histidine or carnosine, with histidine having a superior effect .

As early as 1938 it was reported that carnosine concentrations in skeletal muscle appeared to be higher in so-called 'white muscle' rather than 'red muscle' (Zapp and Wilson 1938). However,

(Davey 1960a) cautioned against a general acceptance of this across species after observing that although many red muscles which had a high myoglobin content had a low concentration of carnosine and anserine, the very red longissimus muscle of dolphins and whales contained very high dipeptide concentrations. A closer and inverse correlation appeared to exist between the oxidative capacity of the muscle, as indicated by its succinate dehydrogenase activity, and the carnosine and anserine content (Davey 1960a). Tamaki *et al.* (1976) dissected out both red and white muscle tissue from gastrocnemius, adductor magnus and latissimus dorsi muscles in rats. He found anserine contents in white muscle tissue of 12 - 17 μmol g^{-1} WW (48 - 68 mmol kg^{-1} DW) in contrast to values of 1 - 2 μmol g^{-1} WW (4 - 8 mmol kg^{-1} DW) in red muscle. Carnosine concentrations in white muscles were approximately two-fold higher than in red muscle (Tamaki *et al.* 1976). Similar findings were described by Suzuki *et al.* (1987b) who reported that the anserine content of tuna white muscle was higher than that of dark muscle. Turnisky and Long (1990) determined carnosine and anserine concentration in rat soleus and gastrocnemius muscles. Carnosine and anserine concentrations in gastrocnemius muscle (58% fast-twitch glycolytic fibres, 37% fast-twitch oxidative-glycolytic fibres) were 7.91 and 5.17 mmol l^{-1} intra-cellular water (ICW), respectively, in contrast to values of 3.65 and 2.27 mmol l^{-1} ICW, respectively in soleus muscle (> 80% slow-twitch oxidative fibres) (Turnisky and Long 1990).

A low but significant correlation ($p < 0.05$) was evident between muscle carnosine concentration and ß*m* (r = 0.69), and between muscle carnosine concentration and the percentage of fast-twitch fibres present (r = 0.46) in human vastus lateralis muscle from untrained subjects and sprint, rowing and marathon trained subjects (Parkhouse *et al.* 1985). However, Mannion *et al.* (1995) reported that there was only a moderate non-significant correlation between carnosine (r = 0.43, $p > 0.05$) or ß*m* (r = 0.42, $p > 0.05$) and percentage type II fibre area in the quadriceps femoris muscle of non-specifically trained subjects.

A similar investigation of gluteus medius muscle from Quarterhorses, Thoroughbreds and Standardbreds established a low but significant ($p < 0.05$) correlation between carnosine concentration and fast-twitch glycolytic fibre percentage (r = 0.53) and a low significant ($p <$

0.05) negative correlation between carnosine and slow-twitch oxidative fibre percentage (r = -0.51) (Bump *et al.* 1989). In a comparison of ß*m* and total imidazole dipeptide concentration (carnosine + anserine) in skeletal muscle of Thoroughbreds, Greyhounds and humans, the higher ß*m* of equine and canine muscle of 117.7 and 105.2 μmol H^+ kg^{-1} DW respectively, in contrast to the human ß*m* of 79.5 μmol H^+ kg^{-1} DW, was explained by the much greater concentration of the imidazole dipeptides in the former. Mean imidazole dipeptide concentrations in equine, canine and human muscle were 108.3, 81.6 and 16.0 mmol kg^{-1} DW. Subtraction of the specific buffering capacity arising from the imidazole dipeptides from ß*m* resulted in almost identical residual values in equine, canine and human muscle of 81.8, 79.1 and 74.2 μmol H^+ kg^{-1} DW, respectively. Therefore, the differences in ß*m* between the three species appeared to arise predominantly from the differences in imidazole dipeptide contents (Harris *et al.* 1990). Similar results have been reported in several other species including, tuna, whale, pig and chicken, where differences in ß*m* between species and muscles were mainly accounted for by differences in the concentrations of histidine and the imidazole dipeptides (Abe *et al.* 1985; Okuma and Abe 1992). Histidine and imidazole dipeptides were found to be major buffering constituents in these species accounting for 12 to 39% of ß*m* (Okuma and Abe 1992).

Multiple linear regression analysis has been used to estimate the carnosine concentrations and ß*m* of type I, IIA and IIB fibres from mixed muscle samples of the gluteus medius at post mortem of the untrained thoroughbred horses and one pony (Table 1.7A). Carnosine showed a strong significant ($p < 0.001$) correlation with ß*m* in mixed muscle samples (r = 0.76) and a significant ($p < 0.001$) correlation with percentage type II fibre section area (r = 0.78). ß*m* was also significantly ($p < 0.01$) correlated with percentage type II fibre section area (r = 0.60). Carnosine concentrations in type I, IIA and IIB fibres were estimated to be 21, 86 and 116 mmol kg^{-1} DW, respectively (Sewell *et al.* 1990). Comparative estimates of carnosine concentrations and ß*m* by multiple linear regression analysis were later made in two-year-old Thoroughbreds actively engaged in training and racing (Table 1.7B). Carnosine concentrations were higher in these horses and were estimated to be 54, 85 and 180 mmol kg^{-1} DW in type I, IIA and IIB fibres, respectively (Sewell *et al.* 1992).

Table 1.7 A Multiple linear regression analysis estimates of the carnosine concentration and ßm of type I, IIA and IIB muscle fibres from untrained thoroughbred horses. Adapted from Sewell *et al.* (1990).

Table 1.7 B Multiple linear regression analysis estimates of the carnosine concentration and ßm of type I, IIA and IIB muscle fibres from two-year-old thoroughbred horses engaged in training and racing.
Adapted from Sewell *et al.* (1992).

A

Fibre type	I	IIA	IIB	SEM
Carnosine (mmol kg^{-1} DW)	21	86	116	16
$ßm$ (mmol H^+ kg^{-1} DW, pH 7.1 - 6.5)	78	98	109	9
$ßm_{carnosine}$	7	29	38	
$ßm_{non\text{-}carnosine}$	71	69	71	

SEM = Standard error of the mean.

B

Fibre type	I	IIA	IIB	SEM
Carnosine (mmol kg^{-1} DW)	54	85	180	15
$ßm$ (mmol H^+ kg^{-1} DW, pH 7.1 - 6.5)	88	98	130	9
$ßm_{carnosine}$	18	28	60	
$ßm_{non\text{-}carnosine}$	70	70	70	

SEM = Standard error of the mean.

1.2.4 Other functions of carnosine

Antioxidant activity

The highest rates of oxidative metabolism in mammalian tissues are found in skeletal muscle and brain. Oxygen utilization in skeletal muscle is greatly increased during exercise and hence there is an increased potential for tissue damage via the generation of oxygen based free radicals and other reactive species (oxidative stress). It has been proposed that at the cellular level carnosine, and other imidazole dipeptides, may function as endogenous antioxidants which protect macromolecules, including DNA, RNA, proteins and membrane lipids, from oxidative stress induced damage caused by reactive oxygen species, such as singlet oxygen, hydroxyl radicals ($^{\bullet}OH$), superoxide radicals ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hypochlorite (^{-}OCl) (Boldyrev *et al.* 1988; Dahl *et al.* 1988; Dupin *et al.* 1984; Kohen *et al.* 1988). As a result of *in vitro* studies utilizing physiological concentrations of carnosine, several different mechanisms have been suggested by which carnosine may function as an antioxidant including free radical scavenging, chelation of divalent metal ions (Brown 1981; Kohen *et al.* 1988) and elimination of pre-formed lipid peroxidation products (Esterbauer *et al.* 1986).

Carnosine and anserine were reported to attenuate *in vitro* Fe^{2+} / ascorbate induced lipid peroxidation in rabbit sarcoplasmic reticulum membranes (Boldyrev *et al.* 1989; Boldyrev *et al.* 1988) and rat serum lipids (Chasovnikova *et al.* 1990) both by chelation of Fe^{2+} ions and by removal of pre-formed peroxidation products. A suppression of stress induced brain and serum lipid peroxidation in rats, following carnosine administration, has also been reported (Gulyaeva *et al.* 1989). Other studies however, have cast doubt on the ability of carnosine to significantly inhibit Fe^{2+} catalysed lipid peroxidation when it was shown that carnosine can interfere with the TBA assay used which can cause an overestimation of the degree of inhibition (Aruoma *et al.* 1989; Salim-Hanna *et al.* 1991). Furthermore, the ability of carnosine to effectively chelate Fe^{2+} ions has recently been refuted following nuclear magnetic resonance (NMR) studies which have shown that carnosine does not form a complex with iron (Decker *et al.* 1992). Carnosine is able to form chelates with Cu^{2+} ions and has been shown to be effective in preventing both Cu^{2+} catalyzed hydroxylation (Kohen *et al.* 1988) and oxidation of deoxyguanosine (Decker *et al.*

1992). Individually, physiological concentrations of carnosine, anserine and homocarnosine have been reported to be capable of scavenging $^{\bullet}OH$ radicals but not $O_2^{-\bullet}$, H_2O_2 and ^{-}OCl (Aruoma *et al.* 1989), although (MacFarlane *et al.* 1991) have suggested that mixtures of carnosine, anserine, homocarnosine and 1-methylhistidine function synergistically to inhibit oxidation by $O_2^{-\bullet}$ *in vitro* . Carnosine has also been shown to be effective at quenching singlet oxygen (Shvachko *et al.* 1990). Conversely, in the presence of Cu^{2+} carnosine is reported to promote the formation of $O_2^{-\bullet}$ but also to catalyse its conversion to H_2O_2 (Hartman and Hartman 1992). It appears therefore, that carnosine can behave as an antioxidant towards some specific reactive oxygen species, but does not function as a broad-spectrum antioxidant.

It has been proposed that the putative antioxidant properties of carnosine, anserine and balenine may protect skeletal muscle tissue from free radical induced damage during oxidative stress (Kohen *et al.* 1988). This hypothesis may in part be supported by the presence of an estimated 21 - 54 mmol kg^{-1} DW of carnosine in equine type I muscle fibres (Sewell *et al.* 1990; Sewell *et al.* 1992), as they have a high oxidative capacity but negligible glycolytic capacity, minimal H^+ production and hence very low buffering requirement.

Regulation of muscle phosphorylase activity

Carnosine has been shown to activate rabbit muscle phosphorylase *a* and *b* *in vitro* in the presence of AMP (Johnson *et al.* 1982). Phosphorylase *a* was also activated in the absence of AMP. The maximal degree of activation (approximately 100%) occurred at a carnosine concentration of 50 m*M* with a greater activation evident for phosphorylase *b*. Anserine activated both phosphorylase *a* and *b* to a lesser extent than carnosine, eliciting a maximal effect (approximately 40%) at 30 - 40 m*M*. However, anserine inhibits phosphorylase *b* at concentrations above 30 m*M*. The relationship between dipeptide concentration and relative phosphorylase activity displayed a biphasic relationship; increasing dipeptide concentrations beyond 50 m*M* for carnosine and 30 m*M* for anserine progressively reduced the relative activity. The inclusion of allosteric inhibitors including ATP and D-glucose in the assay medium attenuated but did not eliminate the activation effect of carnosine on phosphorylase *a* and *b*.

Some slight anserine induced activation of phosphorylase remained in the presence of allosteric inhibitors. Histidine inhibited *a* and *b* forms of the enzyme. ß-alanine inhibited the *a* form and marginally activated phosphorylase *b*. 1-Methylhistidine slightly increased the activity of both forms of phosphorylase (Johnson *et al.* 1982). The activation of rabbit muscle phosphorylase *a* and *b* by carnosine and anserine was later replicated and contrasted with their effect on potato and yeast phosphorylase. Both carnosine and anserine inhibited the activity of potato and yeast phosphorylase at equivalent concentrations to those employed in the muscle enzyme assays. The dipeptides affected the enzyme V_{max} values rather than the K_M values for the substrates. It was surmised from kinetic data that both carnosine and anserine compete for the same binding site(s) on muscle phosphorylase. Furthermore, the contrast in the effects of the dipeptides between the muscle enzyme and those from the potato and yeast sources may be indicative of specific imidazole dipeptide binding sites on muscle phosphorylase (Johnson and Aldstadt 1984).

Myosin ATPase activation

Carnosine (2 - 10 m*M*) was reported to elicit a 30 - 60% activation of the *in vitro* hydrolysis of ATP in the presence of glycerinated rabbit skeletal muscle myofibrils at an ATP concentration of 5 m*M* (Yun and Parker 1965). In a later study *in vitro* , both carnosine and anserine were shown to promote ATP hydrolysis at physiological concentrations mediated by either myosin or glycerinated myofibrils in the presence of K^+, Mg^{2+} and Ca^{2+} ions. Myosin ATPase activity of homogenized myofibrils was increased by 100% in the presence of 20 m*M* carnosine. Anserine produced greater activation than carnosine under the same conditions (Avena and Bowen 1969). Parker and Ring (1970) showed that carnosine had no effect on the myosin ATPase activity of invertebrate muscle and concluded that carnosine induced activation is confined to those muscles which naturally contain high concentrations of the imidazole dipeptides.

Histamine synthesis in response to shock and wound healing

Histamine induced vasodilation and its central role in the mediation of cardiovascular function and circulatory shock has been recognised for many years. More recently feline endotoxic and canine hemorrhagic shock have been associated with increases in plasma and tissue histamine

concentrations (Nagy *et al.* 1986; Parratt *et al.* 1986). Nagy offered no explanation for the source of the increased amount of histamine, however, Parratt observed an increase in histidine decarboxylase activity and postulated that greater histamine concentrations arose from increased histamine biosynthesis in non-mast cell sources.

Histamine is believed to be involved in the biochemical processes of wound healing, although the precise mechanism by which this occurs is unclear (Kahlson and Zenderfeldt 1972). Histamine production, stimulated by the administration of the mast-cell degranulation activator compound 48/80, enhances the healing response (Fitzpatrick and Fisher 1982b). Intra-peritoneal injections of carnosine have also been reported to restore at site wound breaking strength and collagen deposition in histidine deficient rats (Fitzpatrick and Fisher 1982a). Additionally, post-wounding reduction of tissue carnosine concentration was prevented by the intra-peritoneal administration of either histidine or histamine at the time of injury (Fitzpatrick and Fisher 1982b). On the basis of these results it was proposed that a possible metabolic inter-relationship between carnosine, histidine and histamine exists and that this provides a non-mast cell reservoir of histamine, via carnosine hydrolysis to histidine and subsequent decarboxylation, available which is available during periods of increased demand. This hypothesis was investigated in the rat by artificially stimulating mast-cell histamine release by administering compound 48/80. This procedure caused a rapid increase in muscle histamine concentration and histidine decarboxylase activity, which was followed by a sustained increase in carnosinase activity and a reduction in muscle carnosine concentration at 24 h post administration (Greene *et al.* 1984).

The involvement of carnosine in the biosynthesis of histamine and the subsequent effects on the wound healing processes was supported by the later work of Nagai *et al.* (1986) who demonstrated that in rats treated with hydrocortisone to suppress wound healing, subsequent treatment with carnosine lead to improved skin tensile strength at the incision site in contrast to controls. Similar effects were produced with the co-administration of histidine and ß-alanine but not with ß-alanine alone. Increases in both histidine decarboxylase activity and histamine content were found at the wound site following carnosine administration. No significant change

in carnosinase activity was found. It was proposed that the improvement in wound healing was partially attributable to the vasodilatory action of histamine which improved effusion from the wound site during the initial inflammation and also that free ß-alanine arising from carnosine degradation was utilized for collagen synthesis (Nagai *et al.* 1986). The antioxidative properties of carnosine have also been used to explain the apparent efficacy of carnosine in promoting wound healing. Silaeva *et al.* (1990) reported that the topical application of carnosine to experimentally induced wounds in rats reduced the accumulation of lipid peroxidation products with conjugated diene and ketodiene groups and thiobarbituric acid-active products, in contrast to saline treated controls. The time to complete epithelization of the wounds was reduced in the carnosine treatment group in contrast to controls (Silaeva *et al.* 1990).

Possible correlations between the distributions of carnosine, histidine, histamine and 3-methylhistidine in different tissues of the rat, mouse, guinea pig and man have been investigated an higher concentrations of carnosine were often though not exclusively found in those tissues which had higher levels of histamine (Flancbaum *et al.* 1990).

Vasoconstriction

Carnosine was shown to exhibit stereospecific depressor action following intra-venous administration in anaesthetized cats (du-Vigneaud and Behrens 1939; du-Vigneaud and Hunt 1936). Melville *et al.* (1990) have reproduced this finding but also showed a pressor response in anaesthetized and pithed rats. Furthermore, they reported that carnosine exhibited both α-adrenoreceptor agonist and antagonist properties in isolated rabbit saphenous vein (Melville *et al.* 1990). More, recently it has been reported from *in vitro* studies that carnosine chelated with Zn^{2+} ions exhibits sustained vasoconstrictor activity in isolated rabbit saphenous vein and artery. Carnosine concentrations of up to 10 mM were necessary to induce contraction in the absence of Zn^{2+} ions, however, in the presence of 10 μM Zn^{2+} contracture was greatly potentiated, and the concentration of carnosine needed to elicit half of the maximum effect was reduced from 1.4 mM to 15 μM. The presence of zinc alone did not induce contraction. Certain zinc containing metalloproteins, such as angiotensin and Cu-Zn superoxide dismutase are significant in vascular

function and it was postulated that carnosine may potentiate the effects of these proteins by competitively binding zinc ions (O'Dowd *et al.* 1995).

Neurotransmitter or neuromodulator

There has been a steady accumulation of evidence which suggests that carnosine functions as either a neurotransmitter or neuromodulator in the olfactory bulb and epithelium of many vertebrates. It has been shown that carnosine is present in the primary olfactory pathway of the mouse and more specifically to be compartmentalized within the olfactory receptor neurons (Margolis 1974). Carnosine occurs at relatively high concentration in mouse olfactory bulb, 0.9 - 2.2 nmol mg^{-1} WW (3.6 - 8.8 mmol kg^{-1} DW), although these values are much lower than those associated with most mammalian skeletal muscles (Ferriero and Margolis 1975; Neidle and Kandera 1974; Wideman *et al.* 1978). It was also established that carnosine is synthesised *in situ* by carnosine synthetase (Harding and Marshall 1976; Horinishi *et al.* 1978; Ng and Marshall 1978) and that the carnosine synthetase activities in mouse olfactory bulb and epithelium were 92 and 495 pmol mg^{-1} protein h^{-1}, respectively, in comparison with a skeletal muscle carnosine synthetase activity of 37 pmol mg^{-1} protein h^{-1} (Harding and Marshall 1976). Carnosine appears to be transported into regions of the olfactory bulb which contain the olfactory nerve axons and terminals (Burd *et al.* 1980). Carnosine is reported to be catabolised to its precursor amino acids by carnosinase within the olfactory pathway (Harding and Marshall 1976). Carnosinase activity within the epithelium, almost 15000 pmol mg^{-1} protein h^{-1}, was only exceeded by its activity in the kidney (Harding and Marshall 1976). The suggestion that specific carnosine receptors are present in the olfactory pathway was supported by evidence showing that membrane fractions of olfactory bulbs displayed reversible, saturable and stereospecific carnosine binding (Hirsch *et al.* 1978; Hirsch and Margolis 1979). *In vitro* experiments have shown carnosine to be released from olfactory bulb synaptosomes by two processes, the first of which was gradual, spontaneous and non-reliant upon depolarization. The rate of this release was increased two-fold on the addition of 1 m*M* carnosine. The second mechanism was calcium-dependent and depolarization sensitive (Rochel and Margolis 1982). These results were considered to be consistent with the

proposed function of carnosine as a neurotransmitter in the olfactory pathway of the mouse (Rochel and Margolis 1982).

The presence of carnosine in other regions of the CNS including the retina of mammals has also been described (Margolis 1980; Margolis and Grillo 1984). The existence of anserine in the CNS and retina of birds has been reported (Fisher *et al.* 1977; Margolis and Grillo 1984). The cellular distributions of carnosine and anserine in the CNS of vertebrates have been investigated by the use of imidazole dipeptide specific antisera for immunocytochemical localization. In the rat, carnosine immunoreactivity was restricted to neurons, their axons and synaptic terminations in the glomerular layer of the olfactory bulb within the olfactory pathway, and non-olfactory carnosine distribution was confined to astrocytes and cerebellar Bergmann glia. In avian CNS widespread anserine immunoreactivity appeared to be solely associated with glial cells (Biffo *et al.* 1990). The presence of carnosine within the receptor neurons of human olfactory mucosa was also observed using immunohistochemical techniques (Sakai *et al.* 1990).

Activation of muscle calpains

It has been proposed that calpains, a group of calcium activated proteinases present in many types of eukaryotic cells, are involved in the degradation of contractile proteins (Dayton *et al.* 1976). Calpain I and calpain II exhibit maximum activity at micromolar and millimolar Ca^{2+} concentrations, respectively (Birkhold and Sams 1994; Murachi 1985; Suzuki *et al.* 1987a). It was recognised that calpain activity could be regulated by endogenous antagonists and agonists of Ca^{2+} ion binding such as calpastatin, a protein inhibitor of calpain, (Takano *et al.* 1986) and isovalerylcarnitine, an activator of human neutrophil calpain (Pontremoli *et al.* 1987). The high intra-cellular concentrations of carnosine and anserine in skeletal muscle prompted Johnson and Hammer (1989) to investigate a possible regulatory role for these and their constituent amino acids on the activity of calpain II isolated from hamster and chicken smooth muscle . During *in vitro* studies they found that 80 m*M* concentrations of carnosine and anserine increased the activity of chicken and hamster muscle calpain II by approximately 20 - 30% at a Ca^{2+} ion concentration of 2.5 m*M* . 1-Methylhistidine increased the activity of chicken calpain II by about

the same amount and doubled the activity of hamster calpain II. The activation effects of these compounds were markedly attenuated at a Ca^{2+} ion concentration of 5 μM. Calpain II activities were only 10 - 40% of those at 2.5 mM Ca^{2+}. It was also found that anserine and 1-methylhistidine reduced the inhibitory action of calpastatin on calpain II, whereas carnosine increased inhibition. Johnson and Hammer (1989) concluded that carnosine, anserine and 1-methylhistidine were not potent calpain II activators but that synergistically they may influence the interaction between calpain II and calpastatin. However, this effect is likely to be negligible *in vivo* where both imidazole dipeptide and Ca^{2+} ion concentrations are much lower than the *in vitro* concentrations used.

In vitro studies have indicated that calpain II significantly reduces the activity of carnosine synthetase by as much as 60% at a calpain to tissue protein w/w ratio of 0.15 and that probably arises from extensive proteolysis of carnosine synthetase by calpain. It was proposed that such an effect might cause a reduction in muscle carnosine concentration *in vivo* and that this may explain reports of reduced muscle carnosine concentrations as a result of ageing, hypertension, muscular dystrophy and denervation (Johnson and Hammer 1994).

1.2.5 Carnosine metabolism

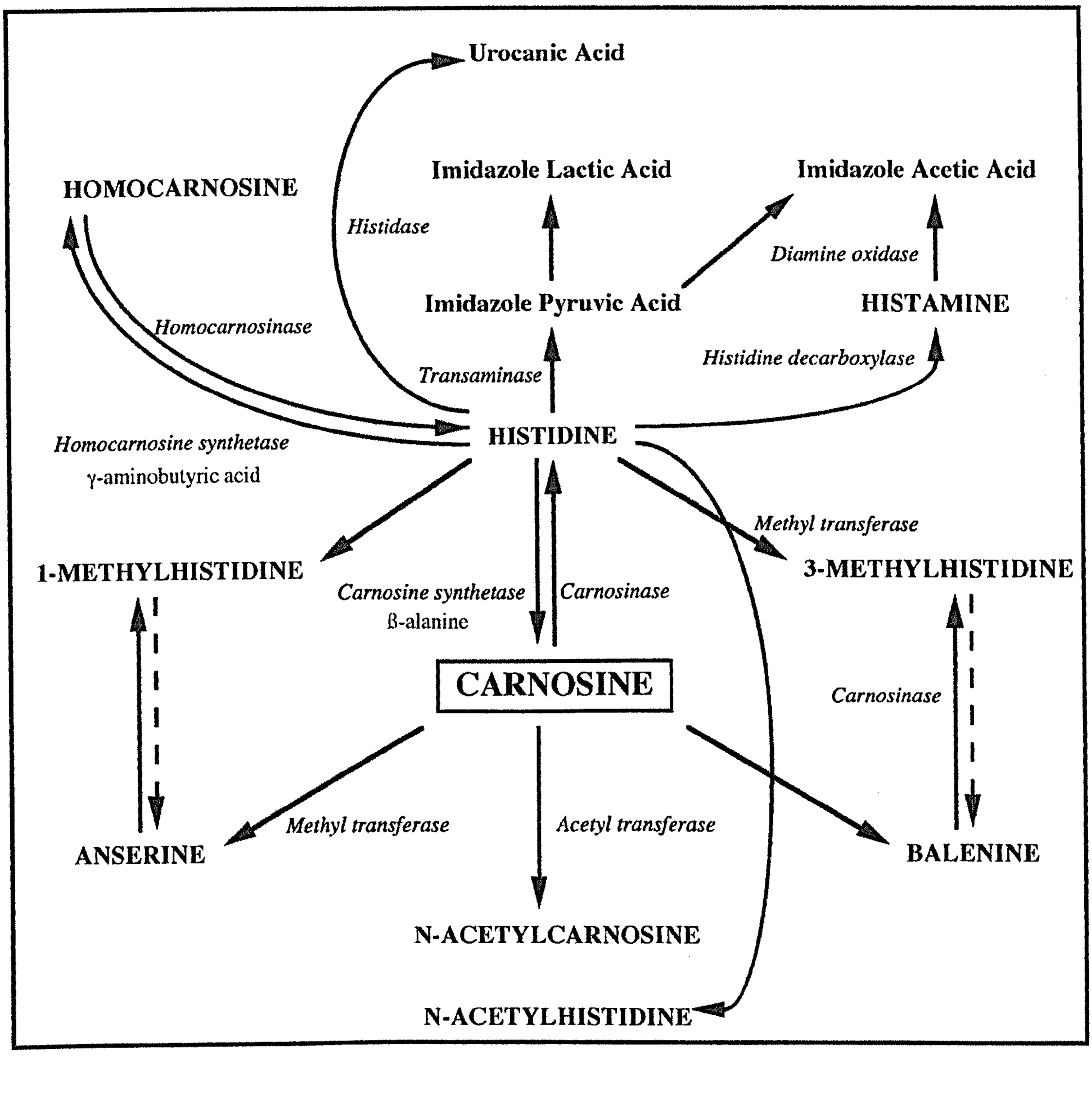
Biosynthesis and carnosine synthetase

Early experimental work involving the use of intact animals and isolated tissue slices provided evidence to support the direct biosynthesis of carnosine from histidine and ß-alanine. Studies involving cell free extracts of chick skeletal muscle demonstrated the existence of an enzyme of broad specificity that catalyses not only the synthesis of carnosine, but also anserine, homocarnosine and several other dipeptides (Kalyanker and Meister 1959; Winnick and Winnick 1959). Similar enzyme regulated synthesis was evident *in vivo* and *in vitro* in frog brain (Yockey and Marshall 1969). Carnosine synthetase, or homocarnosine-carnosine synthetase, was subsequently isolated from rat brain, partially purified and characterized (Skaper *et al.* 1973). Carnosine synthetase (EC 6.3.2.11) which is cytosolic in origin (Ng and Marshall 1978) appears to be a dimer of MW 250,000, with a very high histidine content of 141 per 1000

residues (Rosario *et al.* 1981). The enzyme has an optimum activity at pH 7.4 and a requirement for ATP and Mg^{2+} (Skaper *et al.* 1973). The apparent K_M for histidine was approximately 16 μM (Horinishi *et al.* 1978), whereas that for ß-alanine was 1.0 - 1.8 mM (Kish *et al.* 1978; Ng and Marshall 1978). The enzyme is also responsible for the synthesis of homocarnosine in the CNS and has a K_M for γ-aminobutyric acid of approximately 8.8 mM (Kish *et al.* 1978). It does not catalyse the synthesis of anserine from ß-alanine and 1-methylhistidine, rather anserine is formed via methylation of carnosine (Bauer and Schultz 1994). Carnosine synthetase is inhibited by several ß-alanine analogues, but most effectively by 3-aminopropanesulphonic acid (Seely and Marshall 1982). Carnosine synthetase activities were measured in the 100000 g supernatant fractions of heart, brain, liver and muscle of rat, mouse, chick and frog and were found to be generally highest in skeletal muscle (chick pectoral muscle; 21.4 nmol g^{-1} h^{-1}) and lowest in liver (rat liver 0.1 nmol g^{-1} h^{-1}) (Ng and Marshall 1976a). A similar distribution of activities between tissues was found in all four species studied. High levels of carnosine synthetase activity have also been observed in chick erythrocytes (Ng and Marshall 1976b) and olfactory bulbs (Ng and Marshall 1978).

Intra-muscular carnosine biosynthesis has been demonstrated *in vivo* by the administration of radio-labelled precursor amino acids. Watanabe and Konosu (1979) demonstrated that following the intra-muscular injection of ^{14}C-histidine in the eel, the radio-label was incorporated exclusively into carnosine within the muscle. Similar results using ^{14}C-histidine and ^{14}C-ß-alanine were obtained in tuna (Abe *et al.* 1986), trout (Abe and Hochachka 1987) and rats (Tamaki *et al.* 1980). Furthermore, a greater rate of incorporation of the radio-label into trout muscle carnosine was observed following the administration of ^{14}C-ß-alanine rather than ^{14}C-histidine, suggesting that ß-alanine availability may be a limiting factor in carnosine biosynthesis (Abe and Hochachka 1987). This is supported by data from Tamaki *et al.* (1980) which showed a two-fold increase in the incorporation of ^{14}C-histidine into carnosine following pre-administration of non-radioactive ß-alanine.

Figure 1.3 Metabolic pathways of imidazole dipeptide biosynthesis and degradation.


Urocanic Acid
HOMOCARNOSINE
Imidazole Lactic Acid
Imidazole Acetic Acid
Histidase
Diamine oxidase
Imidazole Pyruvic Acid
HISTAMINE
Homocarnosinase
Transaminase
Histidine decarboxylase
Homocarnosine synthetase
γ-aminobutyric acid
HISTIDINE
Methyl transferase
1-METHYLHISTIDINE
3-METHYLHISTIDINE
Carnosine synthetase
ß-alanine
Carnosinase
CARNOSINE
Carnosinase
Methyl transferase
Acetyl transferase
ANSERINE
BALENINE
N-ACETYLCARNOSINE
N-ACETYLHISTIDINE

Factors influencing biosynthesis and muscle content

The results from several studies in various species indicate that substrate availability, including dietary availability, may be a limiting factor to the regulation of carnosine biosynthesis and accumulation in vertebrate skeletal muscle. Reduced skeletal muscle carnosine concentrations were found in several species, including adult rats (Fuller *et al.* 1947; Quinn and Fisher 1977), eels (Abe and Ohmama 1987), salmon (Luckton and Olcott 1958) and adult roosters (Leveille *et al.* 1960), maintained on histidine-free or histidine deficient diets. Dietary histidine deficiency causes a more rapid decline in skeletal muscle carnosine concentration in young animals, such as chicks (Ousterhout 1960; Ousterhout and Luckton 1960) and rats (Barbaro *et al.* 1978). Conversely, supplementation of the diet with histidine increases in the skeletal muscle carnosine concentration once the histidine requirements for optimum growth have been fulfilled (Robbins *et al.* 1977). Rats fed a diet supplemented with histidine at a level of 5% w/w showed a two-fold greater carnosine concentration in the gastrocnemius muscle in contrast to rats on a control diet with a histidine content of 0.76% w/w (Tamaki *et al.* 1977). Dietary histidine supplementation over two weeks in the mature Quarterhorse, at 0.4% w/w in one study and up to 0.56% w/w twice per day in a further study, produced small but statistically non-significant increases in the carnosine concentration of the middle gluteal muscle (Powell *et al.* 1991; Miller-Graber and Seyers 1993).

No investigations of the influence of dietary ß-alanine supplementation on muscle carnosine content appear to have made. However, twice-daily intra-peritoneal injections of very large doses of ß-alanine (22 mmol kg^{-1} BW, 2000 mg kg^{-1} BW) in adult mice over a 5 day period produced a ten-fold increase in skeletal muscle carnosine concentration in contrast to controls (Margolis *et al.* 1985).

Increasing age appears to result in an initial increase in skeletal muscle imidazole dipeptide concentration during maturation and a subsequent decrease during senescence. The mean muscle imidazole dipeptide concentrations in rat longissimus dorsi increased from 2.49 to 2.99 μmol g^{-1} WW from age 3 to 12 months followed by a reduction to 1.94 μmol g^{-1} WW at 27 months. The

overall trend was due to changes in the anserine concentration as carnosine showed a continued decline from 3 - 27 months. Similar results were evident in the quadriceps femoris (Johnson and Hammer 1992). A similar trend appeared to evolve in human muscle with increasing age from the analysis of 32 muscle samples from subjects aged between 3 months and 80 years. Muscle carnosine concentrations in the younger age group (3 months to 3 years) were 1.5 - 2.5 μmol g^{-1} WW in contrast to values in the teenage group of 4.9 - 8.3 μmol g^{-1} WW. Subjects in the older age group had carnosine values (1.0 - 1.5 μmol g^{-1} WW) lower than the younger group. However, the lower carnosine contents in the older age group were in patients undergoing extended immobilization following hip fracture (Christman 1976).

In experimental rats unilateral ischemic denervation of gastrocnemius muscle caused a significant decline ($p < 0.01$) in the carnosine concentration over a period of three weeks and a slight but non-significant increase in the anserine concentration (Tamaki *et al.* 1976). Carnosine and anserine concentrations in denervated and intact muscles are given in Table 1.8. Turnisky and Long (1990) reported no effect of denervation on the anserine contents of rat soleus and plantaris muscle, a non-significant decrease in the carnosine content of these two muscles, but a significant increase in the muscle concentrations of carnosine ($p < 0.05$) and anserine ($p < 0.005$) in the gastrocnemius muscle. However, these muscles were analysed only three days after denervation, in contrast to three weeks after in the study of Tamaki *et al.* (1976).

Table 1.8 Carnosine and anserine concentrations in denervated and intact rat gastrocnemius muscle. Adapted from Tamaki *et al.* (1976).

Treatment	Weeks post-treatment	Mean concentration ± SEM (mmol kg^{-1} DW)	
		Carnosine	Anserine
Non treated rats	0	22.3 ± 3.9	10.2 ± 2.7
	3	28.9 ± 0.9	18.2 ± 2.7
Treated rats			
Intact side	1	29.6 ± 1.6	12.1 ± 0.9
	2	24.7 ± 1.1	10.8 ± 2.0
	3	24.4 ± 3.9	15.8 ± 1.6
Denervated side	1	17.0 ± 3.8†	15.7 ± 2.9
	2	5.9 ± 0.8††	15.3 ± 2.1
	3	3.6 ± 0.7††	18.5 ± 1.9

SEM = Standard error of the mean.

† = $p < 0.05$ compared to intact side.

†† = $p < 0.01$ compared to intact side.

Intestinal absorption and transport mechanisms

Experimental evidence has been provided for the existence of peptide specific transport mechanisms within the brush border membranes of the small intestine (Craft *et al.* 1968; Matthews 1975). Following absorption, carnosine, like many other peptides, was thought to be hydrolysed in the cytoplasm of the intestinal epithelium prior to transfer of the resulting amino acids into the portal blood. Carnosine administration to human subjects by ingestion or jejunal perfusion resulted in a rapid increase in circulatory concentrations of histidine and ß-alanine, but no appearance of carnosine in the blood (Asatoor *et al.* 1970; Cook 1976). Carnosine and anserine were absorbed intact from rat intestine both *in vitro* and *in vivo* (Hama *et al.* 1976). Large increases in rat blood carnosine and anserine concentrations occurred two hours after forced feeding of large doses (up to 4500 mg kg^{-1} BW) of the imidazole dipeptides (Hama *et al.* 1976). This finding was later reproduced although it was suggested that part of the orally administered carnosine was hydrolysed to histidine and ß-alanine in the small intestine (Tamaki *et al.* 1985). However, more recent investigations have supported the hypothesis that a significant proportion of the intact peptides are transported into the circulation (Gardner and Wood 1989). Plasma carnosine (81.8, 44.4 and 4.6 μM) was detected in one subject 30, 60 and 90 min after administration of 4 g of carnosine in both isotonic and hypertonic test solutions containing lactulose, when sample collection and processing were performed at 4°C (Gardner *et al.* 1991).

In vitro studies provided evidence for the active transport of carnosine by hamster jejunum (Matthews *et al.* 1974). Nutzenadel and Scriver (1976) reported active transport of carnosine by a high K_M system (5 - 10 mM) in rat intestine which discriminated it from ß-alanine, α-amino acids and other dipeptides, and that intra-cellular hydrolysis prevented concentrative uptake despite the extent of hydrolysis being 10% only in eneterocytes. Carnosine transport in rabbit intestinal brush-border membrane vesicles was optimized by an inward proton gradient with an external pH of 5.5 - 6.0 against a fixed internal pH of 7.5 (Ganapathy and Leibach 1983). *In vitro* carnosine transport in rabbit kidney and mouse and guinea pig intestine conformed to

Michaelis-Menten kinetics, and has been shown to be a carrier mediated Na^+-independent process (Ganapathy and Leibach 1983; Himuki 1985; Rajendran *et al.* 1984).

Catabolism and carnosinase

Historically the investigation of enzyme catalysed hydrolysis of carnosine has been confused by the existence of at least two different forms of the enzyme and complicated by differences in the behaviour of each form in tissues from different species. (Hanson and Smith 1949) reported the existence in pig kidney of 'carnosinase', a specific enzyme responsible for catalysing the hydrolysis of the peptide bond in carnosine to yield its constituent amino acids, histidine and ß-alanine. The enzyme was activated by the presence of Mn^{2+} ions. Carnosinase activity was detected in several tissues including kidney, liver and spleen of both pigs and rats, and a partially purified form of the enzyme was isolated from pig kidney and shown to be a metallo-protein activated by Mn^{2+} and Zn^{2+} ions (Rosenberg 1960; Wood 1957) Subsequently, two electrophoretically different forms of carnosinase were isolated from human liver, kidney and spleen. At pH 8.0 both exhibited the same substrate specificity and were approximately the same size (MW 90000) (Murphey *et al.* 1972). The existence of two carnosine-splitting enzymes was also described in hog kidney (Lenney 1976; Wolos *et al.* 1978). One of these two enzymes was later described as 'homocarnosinase' (Lenney *et al.* 1977). A Mn^{2+} - independent carnosinase and a Mn^{2+} - dependent carnosinase were isolated from mouse kidney (Margolis *et al.* 1979; Margolis *et al.* 1983). Rat brain was also shown to contain two carnosine hydrolysing enzymes; carnosinase and ß-Ala-Arg hydrolase (Kunze *et al.* 1986). Studies in mouse olfactory tissues and rat liver to establish the subcellular distribution of carnosinase have indicated that the majority of the activity is localized in the cytoplasm (Harding and Marshall 1976; Kalra *et al.* 1988). Human tissue carnosinase (MW 90000) was isolated from kidney and characterized as a metallo-cysteine dipeptidase. The enzyme showed optimum activity at pH 9.5, had a p*I* of 5.6, and a K_M (carnosine) of 10 m*M*. It was activated by dithiothreitol (DTT) and *p* -hydroxymercuribenzoate (*p* HMB), was present in all tissues studied and had a wider specificity than hog kidney carnosinase. The enzyme appeared to be identical to prolinase, a non-specific peptidase (Lenney *et al.* 1985).

With more detailed analysis of the properties of hog kidney carnosinase Lenney (1990) attempted to rationalize characterization and nomenclature of carnosinase. He confirmed the presence of two dipeptidases in hog kidney capable of hydrolysing carnosine. He concluded that one was identical to the classical "carnosinase" described originally by (Hanson and Smith 1949), that it also fulfilled the requirements previously ascribed to "homocarnosinase" and "Mn^{2+} - independent carnosinase", and proposed that it be regarded as carnosinase. The second dipeptidase was functionally equivalent to "human tissue carnosinase", "Mn^{2+} - dependent carnosinase" and "ß-Ala-Arg hydrolase", and that it be considered a non-specific dipeptidase. Both carnosinase and non-specific dipeptidase were cytosolic (Lenney 1990).

The abnormal appearance of carnosine in the serum of two juvenile patients suffering from neurological disease and mental retardation prompted Perry *et al.* (1968) to investigate possible carnosinase activity in serum from these patients and in normal healthy subjects. The use of a previously published assay for tissue carnosinase activity indicated a mean enzyme activity of 0.71 and 1.22 μmol ml^{-1} h^{-1} in children and adults, respectively (Perry *et al.* 1968). Serum carnosinase from humans has been isolated (Lenney *et al.* 1982). A glycoprotein with a sub-unit MW of 75000 and a p*I* of 4.4, it was found to be structurally and functionally distinct from both carnosinase (tissue) and non-specific dipeptidase. Activities of serum carnosinase in tissues were measured and were proportional to the amount of trapped blood present. Brain however, contained 9 times more activity than could be accounted for by trapped blood (Jackson *et al.* 1991). It had previously been shown that CSF contained relatively high activities of serum carnosinase (Lenney *et al.* 1983). It was therefore proposed that serum carnosinase is synthesized in brain and transported to the blood via the CSF rather than through the blood-brain barrier. Serum carnosinase activity was also found in six higher primates but not in twelve non-primate mammals except for Golden hamster (Jackson *et al.* 1991). A comparison of the properties of carnosinase, non-specific dipeptidase and serum carnosinase is given in Table 1.9.

Table 1.9 Properties of rationalized carnosinase, non-specific dipeptidase and serum carnosinase. Adapted from Lenney (1990).

Characteristics		Enzyme		
		Carnosinase	Non-specific dipeptidase	Serum carnosinase
Specificity	*carnosine*	100	100	100
	anserine	70	0	88
	homocarnosine	47	0	11
pH optimum		8.8	8.8	8.3
K_m	*carnosine*	0.4 m*M*	5.0 m*M*	
Activators		Mn^{2+}, DTT	Mn^{2+}, DTT	Mn^{2+}
Stabilizers	*@ 50°C*	Mn^{2+}, DTT	Mn^{2+}, DTT	
% Inhibition	*bestatin*	0	50	39
	pHMB	52	89	8
	DTT	-	-	94
MW		57000	70000	75000
p*I*		5.5	5.0	4.4
Active	*without* Mn^{2+}	yes	no	yes
	without DTT	yes	no	
Location		kidney	kidney, liver, brain	Serum, brain, CSF
Equivalent enzymes		"Carnosinase" Hanson and Smith (1949)	Human tissue carnosinase Lenney *et al.* (1985)	
		"Homocarnosinase" Lenney *et al.* (1977)	Mn^{2+} - dependent carnosinase Margolis *et al.* (1983)	
		Mn^{2+} - independent carnosinase Margolis *et al.* (1979)	ß-ala-arg hydrolase Kunze *et al.* (1986)	

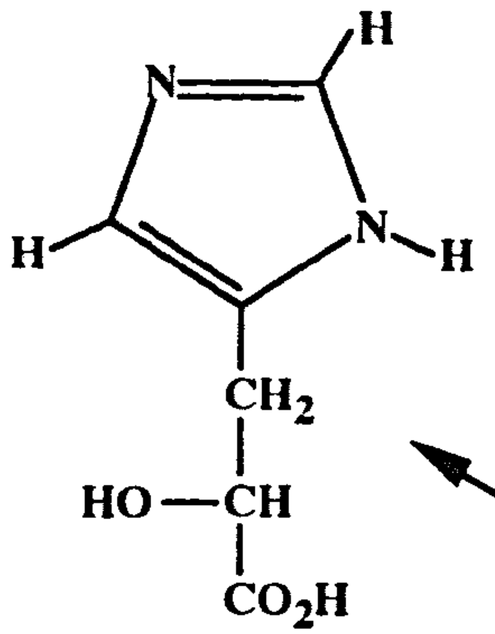
The primary metabolites of carnosine, ß-alanine and histidine, can be further metabolized. The deamination of ß-alanine to malonate semialdehyde is catalyzed by ß-alanine-α-ketoglutarate transaminase. Histidine can be further metabolized as shown in Figure 1.4.

Excretion

Normal urinary excretion of carnosine in humans is relatively high in the neonate but decreases during the first four years. Older children and adults excrete less than 4% of the amount excreted by young children. This urinary excretion pattern appears to be attributable to changes in serum carnosinase activity (Roesel *et al.* 1986).

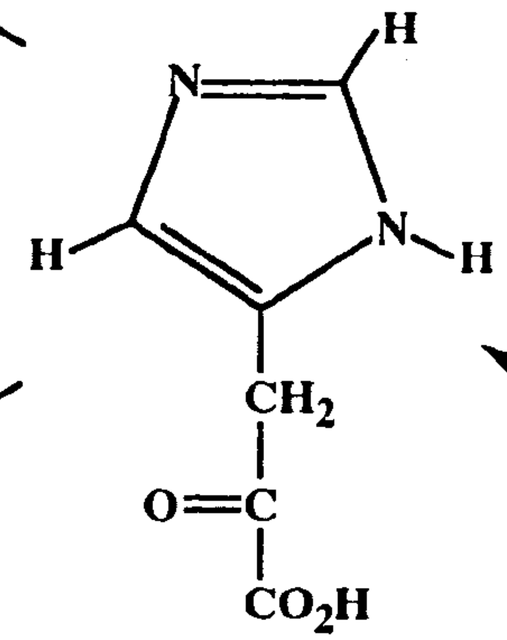
Detection of ophidine (balenine) carnosine and anserine in human urine following meals comprising whale meat, other meats and tuna, respectively, has been reported (Abe *et al.* 1993; Undrum *et al.* 1982). Large amounts of carnosine were detected in the urine of fasted human subjects up to 3 - 5 h after oral administration of 4 g (approximately 18 mmol) of carnosine in both isotonic and hypertonic test solutions containing lactulose. As much as 14% of the dose was recovered in the urine during the subsequent 5 h. Urinary excretion of carnosine was lower following administration in hypertonic solution. Urinary excretion of carnosine was significantly correlated to plasma carnosinase activity ($p < 0.005$, $r = -0.815$) (Gardner *et al.* 1991).

Figure 1.4 Metabolic pathways of histidine catabolism.



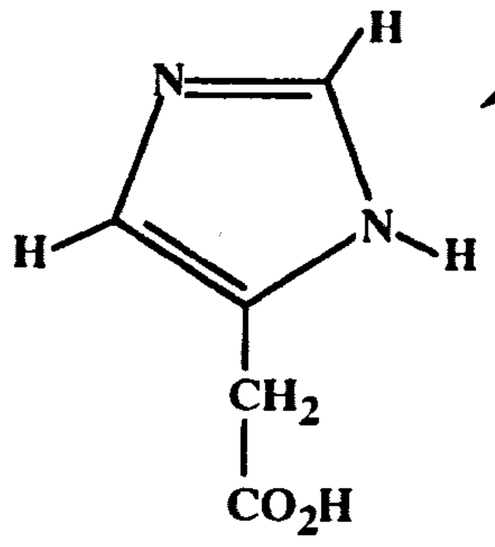
IMIDAZOLE LACTIC ACID

Reduction



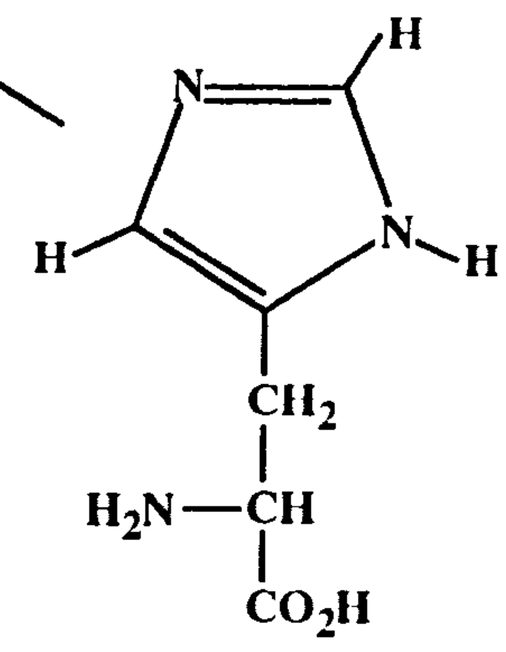
IMIDAZOLE PYRUVIC ACID

Reduction



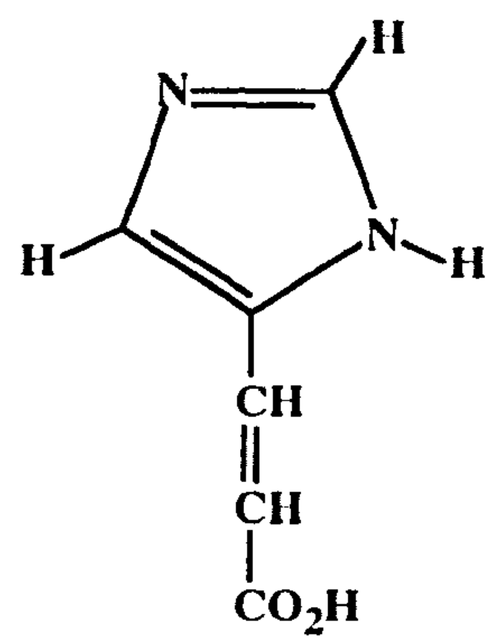
IMIDAZOLE ACETIC ACID

Transamination



HISTIDINE

Deamination Histidase



UROCANIC ACID

1.2.6 Disorders of carnosine metabolism

Hyper-ß-alaninemia

Hyper-ß-alaninemia and ß-aminoaciduria associated with somnolence and seizures was reported and was postulated to arise from a deficiency in the activity of ß-alanine-α-ketoglutarate transaminase . Increased ß-alanine concentrations in brain, liver, kidney and skeletal muscle and large increases in the concentration of carnosine in the brain and muscles, but not in the liver and kidneys were found. Deltoid and abdominal muscle carnosine concentrations, relative to leucine, were 45.0 and 36.1 μmol g^{-1} WW (approximately 180.0 and 144.4 mmol kg^{-1} DW), respectively, in contrast to a control subject where concentrations in the same muscles were 6.84 and 6.62 μmol g^{-1} WW (approximately 27.4 and 26.5 mmol kg^{-1} DW), respectively. These data suggested that increased substrate availability is the probable cause of the increased muscle carnosine content (Scriver *et al.* 1966).

Carnosinemia

Carnosinemia associated with carnosinuria, progressive neurologic disease, severe mental deficiency and myoclonic seizures was reported after excessive urinary excretion of carnosine was encountered in two juvenile patients (Perry *et al.* 1967). Carnosine was detected in fasting plasma from one patient after a normal diet (8 μM) and persisted for at least 72 h after the imposition of a meat-free diet (4 μM). Relatively large amounts of carnosine were excreted in the urine of both subjects (0.97 and 0.25 μmol mg^{-1} creatinine) 72 h after the imposition of the meat-free diet. Carnosine was not detected in fasting plasma from 120 healthy control subjects. A metabolic challenge imposed on both subjects by the administration of a chicken breast diet resulted in an increased excretion of carnosine and the appearance of large quantities of anserine (2.27 and 8.59 μmol mg^{-1} creatinine) in the urine. A greatly elevated carnosine concentration (45.3 μM) and the presence of large quantities of anserine (102 μM) were also detected in the plasma of the patient which had previously exhibited carnosinemia. Neither patient excreted 1-methylhistidine, the normal urinary metabolite of anserine. Carnosine loading tests were performed on both individuals by oral administration at 5 mg kg^{-1} body weight (BW) (22 μmol kg^{-1} BW). At 4 h post administration 15% of the dose had been excreted by the patient exhibiting

both carnosinemia and carnosinuria and 9% by the patient displaying only carnosinuria. The same dose given to a normal healthy subject resulted in the excretion of only 0.4% of the dose within 4 h. Perry *et al.* (1967) proposed the carnosinemia and carnosinuria occurred as a result of impaired degradation of imidazole dipeptides arising from a genetically derived deficiency in carnosinase activity.

The significance of plasma carnosinase activity in the aetiology of carnosinemia in these two patients was later investigated by Perry *et al.* (1968). Plasma carnosinase activities in the two patients were very low, 0.02 ± 0.02 and 0.04 μmol ml^{-1} h^{-1}, with the lower activity present in the patient displaying both carnosinuria and carnosinemia. In contrast, the activities in normal healthy adults and children were 1.21 ± 0.52 and 0.71 ± 0.31 μmol ml^{-1} h^{-1}, respectively. Significant tissue carnosinase activity was found in post mortem extracts of liver, kidney and heart of one patient (Perry *et al.* 1968).

Similar findings to these were later reported, for a family in which two boys with progressive neurological disorders and their physically and mentally normal sister exhibited carnosinuria, by Murphey *et al.* (1973). Carnosinemia was evident in both boys with serum carnosine concentrations of 200 - 300 μM , but not in the sister. Carnosinuria was evident in all three children with carnosine excretion higher in the sister (312 μmol d^{-1}) than in the brothers (63 and 204 μmol d^{-1}). Serum carnosinase activities were < 0.03 μmol ml^{-1} h^{-1}. All three subjects excreted 1-methylhistidine when fed a meal comprising chicken breast, which indicated an unimpaired ability to catabolise anserine (Murphey *et al.* 1973). Electrophoresis of extracts of post mortem liver, kidney and spleen tissue from one of the brothers indicated that one of the two electrophoretic forms of carnosinase was absent and that the one present corresponded to the slower form of the enzyme and exhibited normal activity. Murphey *et al.* (1973) postulated that the absence of serum carnosinase activity in the physically and mentally normal sister precluded a causal relationship between serum carnosinase deficiency and neurological disease. Reduced serum carnosinase activity has also been reported in urea cycle defects which in some cases correlated with carnosinemia (Burgess *et al.* 1975), liver cirrhosis, hepatoma and chronic

hepatitis (Bando *et al.* 1986), Parkinson's disease and multiple sclerosis (Wassif *et al.* 1994), Duchenne muscular dystrophy (Bando *et al.* 1984) and alcoholic chronic skeletal muscle myopathy (Duane and Peters 1988).

Homocarnosinosis

Homocarnosinosis characterized by an increased homocarnosine concentration in cerebrospinal fluid (CSF) and brain, serum carnosinase deficiency and carnosinuria often associated with progressive mental retardation, spastic paraplegia and retinal pigmentation was first described in a 37 year-old woman (Gjessing and Sjaastad 1974). Her two brothers displayed increased CSF homocarnosine concentrations and similar clinical symptoms, her mother showed no neurological abnormalities but had elevated CSF homocarnosine concentrations, and the father, other sibling and two maternal aunts were physiologically, biochemically and mentally normal (Sjaastad *et al.* 1976). CSF homocarnosine concentrations in the mother, two brothers and daughter were 48-75 μM in contrast to the unaffected father and second daughter (0.5 - 2.0 μM). The normal range was 0.9 ± 0.1 to 1.8 ± 1.8 μM (Perry *et al.* 1975; Perry *et al.* 1982). Homocarnosine concentration in the brain of one brother was four-fold higher than in controls. The mother and three children all showed a marked elevation of carnosine excretion on a meat-free diet and after oral carnosine administration, minimal excretion of 1-methylhistidine following a chicken meal and oral anserine administration. These findings were consistent with a deficiency in serum carnosinase activity. Subsequent analysis of carnosinase activity in the four homocarnosinosis patients indicated zero or minimal activity present in both serum and CSF, in contrast to both controls and the unaffected father and daughter (Lenney 1985; Lenney *et al.* 1983). These findings were identical with those associated with carnosinemia and a deficiency in serum carnosinase activity was probably responsible for the increased homocarnosine concentrations evident in homocarnosinosis patients (Lenney *et al.* 1983). A causal relationship between homocarnosinosis, impaired serum carnosinase activity and neurological disorder was considered unlikely (Gjessing *et al.* 1990). No homocarnosinosis was found in twenty-five patients afflicted with various forms of spastic paraplegia (Sjaastad *et al.* 1977).

Other disorders

Increased urinary excretion of carnosine, has been reported in Juvenile Amaurotic idiocy (Stengel/Batten/Vogt/Spielmeyer disease) (Bessman and Baldwin 1962; Levenson *et al.* 1964). Raised homocarnosine concentrations were reported in some untreated cases of Phenylketonuria (Føllings disease) (Sande *et al.* 1970). Reduced carnosine concentration in human lens was correlated with the severity of senile cataracts (Boldyrev *et al.* 1987).

1.3 Objectives

The principal objectives of this research were as follows:

i To develop and validate sensitive, selective and accurate analytical methods for the determination of the concentrations carnosine and other associated biogenic imidazoles in equine tissues and fluids. (Chapter 3)

ii To determine the normal concentration range of carnosine and associated compounds in equine plasma, and to investigate the effects of normal feeding and exercise. (Chapter 4)

iii To determine by direct measurement carnosine concentrations in type I, IIA and IIB fibres from normal and abnormal equine skeletal muscle, and to investigate the effect of training. (Chapter 5)

iv To measure carnosine concentrations in other equine tissues. (Chapter 5)

v To study aspects of carnosine metabolism, transport and excretion in vivo. (Chapter 6)

vi To examine the effectiveness of oral carnosine or histidine and ß-alanine administration in increasing endogenous skeletal muscle carnosine concentrations. (Chapter 7)

CHAPTER 2

GENERAL METHODOLOGY

2.1 SAMPLE COLLECTION AND PREPARATION PROCEDURES

2.1.1 Blood collection

Single blood samples were obtained by venepuncture using disposable sterile (1.2 mm i.d. x 40 mm) 'Monoject' needles and 10 ml plastic syringes (Sherwood Medical, Balmoney, N. Ireland).

Serial blood samples were collected via an indwelling catheter. A 2 cm^2 area over the left jugular vein was scrubbed and shaved. Following local anaesthesia with Xylocaine (0.5 ml 2%) administered subcutaneously, an 'Intraflon 2' (2.4 mm i.d. x 120 mm) Teflon trocart catheter (Vygon UK Ltd., Cirencester, UK.) was inserted, closed with a three-way tap and sutured in place. The catheter was flushed with physiological saline (10 ml) containing 5000 IU l^{-1} heparin (CP Pharmaceuticals Ltd., Wrexham, UK.) as anticoagulant. Blood (5 ml) was drawn through the catheter and discarded immediately prior to collecting each 10 ml blood sample. The catheter was flushed with saline (5 ml) after each sample withdrawl. Blood was collected into 5 ml lithium heparin tubes (LIP Ltd., Shipley, UK.) and stored in ice prior to centrifugation (2000 g for 4 min at 4°C). Plasma was aspirated and stored at -20°C prior to analysis.

2.1.2 Urine collection

Twelve-hour urine collections from both fillies and geldings were performed by the free-flow technique using a collection harness (Harris and Snow 1988). Individual samples were retained in polythene bags suspended under the harness. Samples were mixed thoroughly, the volume recorded, and a 20 ml aliquot taken and stored at -20°C prior to analysis.

2.1.3 Muscle sampling

Muscle samples were collected both from live experimental animals using the percutaneous biopsy technique and by dissection at *post mortem* from euthanased horses which had failed to respond to treatment for a variety of chronic orthopaedic conditions. All animals, with one exception, (see Chapter 4) had no clinical history of muscle disorders or significant weight loss.

Biopsy sampling

Percutaneous muscle biopsies were collected from the right gluteus medius muscle at a point one third of the distance along a line running from the tuber coxae to the head of the tail using a 5 mm Bergström-Stille biopsy needle (Bergström 1962; Snow and Guy 1976). The biopsy site was shaved and disinfected prior to local anaesthesia with 2% Xylocaine^{TM} (1 - 2 ml). The biopsy needle was introduced through a small stab incision made through the skin and muscle fascia with a scalpel blade. Biopsies were taken at depths of 6 cm. Biopsy samples were blotted on filter paper to remove excess blood, placed in a screw-cap tube, frozen in liquid nitrogen (-196^{o}C) and subsequently freeze-dried. Freeze-dried samples were stored in liquid nitrogen.

Post mortem sampling

Tissue samples were collected as quickly as possible, and always within 90 min of euthanasia. The entire left or right middle gluteal muscle was removed and a 1 cm-thick transverse section was dissected from the intact muscle at the mid-point and at points one quarter of the muscle length from each end. Triplicate tissue samples approximately 5 mm^2 by 20 mm were dissected from 9 pre-determined sites (Figure 2.1) such that the fibre orientation was parallel with the long edge. Samples were blotted on filter paper to remove excess blood, stretched slightly to straighten the fibres, frozen in liquid nitrogen (-196^{o}C) and subsequently freeze-dried. Freeze-dried samples were stored in liquid nitrogen.

Muscle powdering

Freeze-dried muscle was allowed to reach ambient temperature in sealed tubes before powdering. The outer surfaces of the samples were removed in order to minimize the contamination from dried blood. The remaining muscle was coarsely powdered with an agate pestle and mortar and finely powdered by drawing the tissue between the serrated tips of sharp curved forceps. Contaminating connective tissue and blood were removed as far as possible. Powdered muscle was stored in liquid nitrogen.

2.1.4 Sampling of other tissues

Other tissue samples comprising liver, kidney, lung, spleen, diaphragm, heart (myocardium), brain (medulla and cerebellum) and gastro-intestinal tract (small intestine, colon, stomach and rectum) were prepared and stored using the procedures described previously for muscle.

2.2 SAMPLE EXTRACTION PROCEDURES

2.2.1 Plasma extraction

Several plasma extraction procedures were employed; the exact procedure used being dependent on the particular analytes to be determined. Samples for carnosine, histidine, ß-alanine and taurine determination were deproteinized and extracted with sulphosalicylic acid, as described in detail in Chapter 3. Samples for N-α-acetylcarnosine determination were deproteinized and extracted with perchloric acid/phosphoric acid, as described in detail in Chapter 3.

2.2.3 Urine extraction

Frozen urine samples were thawed at 37°C and mixed thoroughly. Samples were vortex-mixed for one minute to re-suspend any particulate material immediately prior to taking a 1 ml aliquot for extraction.

2.2.4 Muscle extraction

Powdered muscle (8 - 10 mg) was extracted with aqueous 0.5 *M* perchloric acid containing 1 m*M* EDTA (100 μl mg^{-1}), as described previously (Harris *et al.* 1974). Extracts were vortex-mixed at 2 min intervals over a total period of 15 min, or until the floating muscle particles sink. During mixing intervals samples were stored on ice. Extracts were centrifuged for 5 min at 12000 g at ambient temperature. Aliquots of the acid extracts, for the determination of low concentrations, were stored at -20°C prior to further extraction and analysis. The remaining volumes of the extracts were neutralized with aqueous 2.1 *M* potassium hydrogen carbonate and centrifuged. The supernatants were stored at -20°C prior to analysis. Neutralized supernatant fluid (1 ml) was equivalent to 10 mg of freeze-dried muscle.

2.3.5 Extraction of other tissues

Powdered tissue samples were extracted with perchloric acid as described previously (Harris *et al.* 1974). Acid extracts were stored at -20°C until analysis.

2.3 INDIVIDUAL MUSCLE FIBRE PREPARATION, WEIGHING AND EXTRACTION PROCEDURE

2.3.1 Dissection

A thin longitudinal slice was cut from the freeze-dried muscle sample whilst ensuring that the long edge was parallel with the fibre bed orientation. The thin muscle slice was placed in an agate mortar and compressed gently with the pestle. Gentle rocking of the pestle helped to partially separate the muscle fasciculi. Further separation of the fasciculi was obtained using a pair of dissection needles. One fasciculus was transferred to a pre-cleaned (IMS followed by acetone) microscope slide. With the aid of a stereo dissection microscope and a pair of very fine dissection needles (0.1 mm sharp-point ophthalmic suture needles), individual muscle fibres were separated. Between 30 and 50 fibres per sample were dissected. Separated fibres were stored overnight at -85°C.

2.3.2 Mounting and weighing

Each of three pre-cleaned microscope slides were engraved with a small central grid of 25 squares and labelled with the identity of the horse, the sample No. (i.e. MG3, MG4 etc.), the horse colour code label and the slide number (Figure 2.2).

One individual fibre was removed from the flip-top Eppendorf tube and the tube was re-capped. Three small fragments were cut from one end of the fibre. A small drop of water was placed in the first square of the grid on each slide. One fragment was placed in the first grid-square on each slide. The remaining larger fibre portion was transferred on a microscope slide to a pre-calibrated quartz-fibre fish-pole balance for weighing (Lowry and Passoneau 1972). The cover glass was removed from the glass barrel of the balance and the a dissection needle was used to

transfer the muscle fibre to the end of the quartz fibre. The muscle fibre is held by static attraction. The cover glass was replaced and the deflection recorded using a graticule eyepiece. The muscle fibre was removed from the balance and placed in a 200 μl glass micro-vial which was immediately capped. The above procedures were repeated until 25 fibres were mounted in triplicate and weighed. Completed slides were stored at -20^{o}C prior to histochemical staining. Fibres for extraction were stored at -85^{o}C.

2.3.3 Metabolite extraction

Individual muscle fibres were extracted as described in detail in Chapter 3.

2.4 HISTOCHEMICAL STAINING OF INDIVIDUAL MUSCLE FIBRES.

Freeze-dried individual muscle fibre fragments and frozen muscle sections were characterized as type I, type IIA or type IIB by histochemical staining for myosin ATPase activity by a modification of a published method (Brook and Kaiser 1970).

2.4.1 Preparation of reagents

Sodium acetate solution (0.2 *M*):	Sodium acetate trihydrate (27.22 g) was dissolved in distilled de-ionised water (1000 ml).
Acetic acid solution (50% v/v):	Glacial acetic acid (50 ml) was diluted with distilled de-ionised water (50 ml).
Glycine/calcium chloride buffer:	Glycine (3.00 g) and calcium chloride dihydrate (2.94 g) were dissolved in distilled de-ionised water (1000 ml).
Potassium hydroxide solution (0.1 *M*):	Potassium hydroxide (5.60 g) was dissolved in distilled de-ionised water (1000 ml).
Calcium chloride solution (2% w/v):	Calcium chloride dihydrate (10.00 g) was dissolved in distilled de-ionised water (500 ml).

Cobalt chloride solution (1% w/v):	Cobalt chloride hexahydrate (5.00 g) was dissolved in distilled de-ionised water (500 ml).
Ammonium sulphide solution (1% w/w):	20% w/w ammonium sulphide solution (1 ml) was diluted with distilled de-ionised water (19 ml).

2.4.2 Staining procedure

The pH meter (Corning pH/ion meter, CIBA-Corning) was calibrated between pH 7.0 and 10.0 using a two-point calibration. Glycine/calcium chloride solution (~ 45 ml) was added to two Coplin jars which were incubated at 20°C. One solution was subsequently titrated to pH 9.6 and the other to pH 9.8 using 0.1 *M* Potassium hydroxide. ATP disodium salt (75 mg) was added to the glycine/calcium chloride buffer at pH 9.8 and the solution re-titrated to pH 9.6. The pH meter was re-calibrated between pH 4 .000 and 7.000 using a two-point calibration. Sodium acetate solution was incubated, in a Coplin jar at 20°C and susequently titrated to precisely pH 4.500 with acetic acid (50% v/v). Solutions of calcium chloride (2% w/v) and cobalt chloride (1% w/v) were incubated at 20°C. Two slides of mounted fibres were incubated in the sodium acetate /acetic acid buffer for exactly 5 min, rinsed briefly four times in distilled water and once in the glycine/calcium chloride solution for 30 s prior to incubation in the ATP/glycine/calcium chloride solution for 30 min. After rinsing the slides were incubated in the calcium chloride solution for 90 s, rinsed, incubated in cobalt chloride for 3 min, rinsed and flooded with the ammonium polysulphide solution for 2 min. The slides were rinsed, coverslips were mounted on the slides using UV free aqueous mountant and the edges of the coverslips sealed using clear nail varnish. The stained fibres were viewed using low power microscopy using and characterized as type I, IIA or IIB according to stain intensity; type I fibres stained black, type IIA fibres white and type IIB fibres grey.

Figure 2.1 Muscle sampling sites within the middle gluteal muscle.

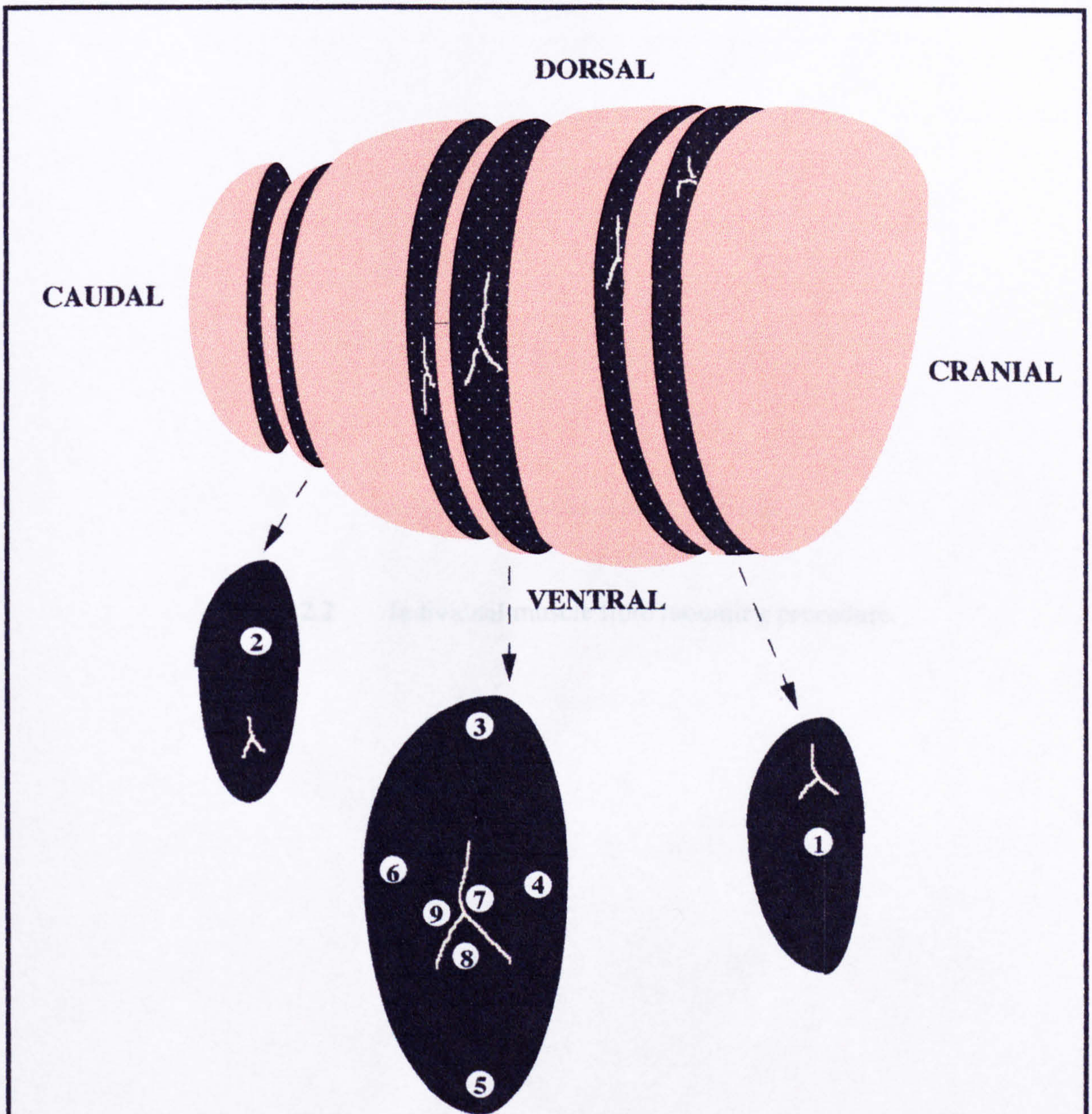
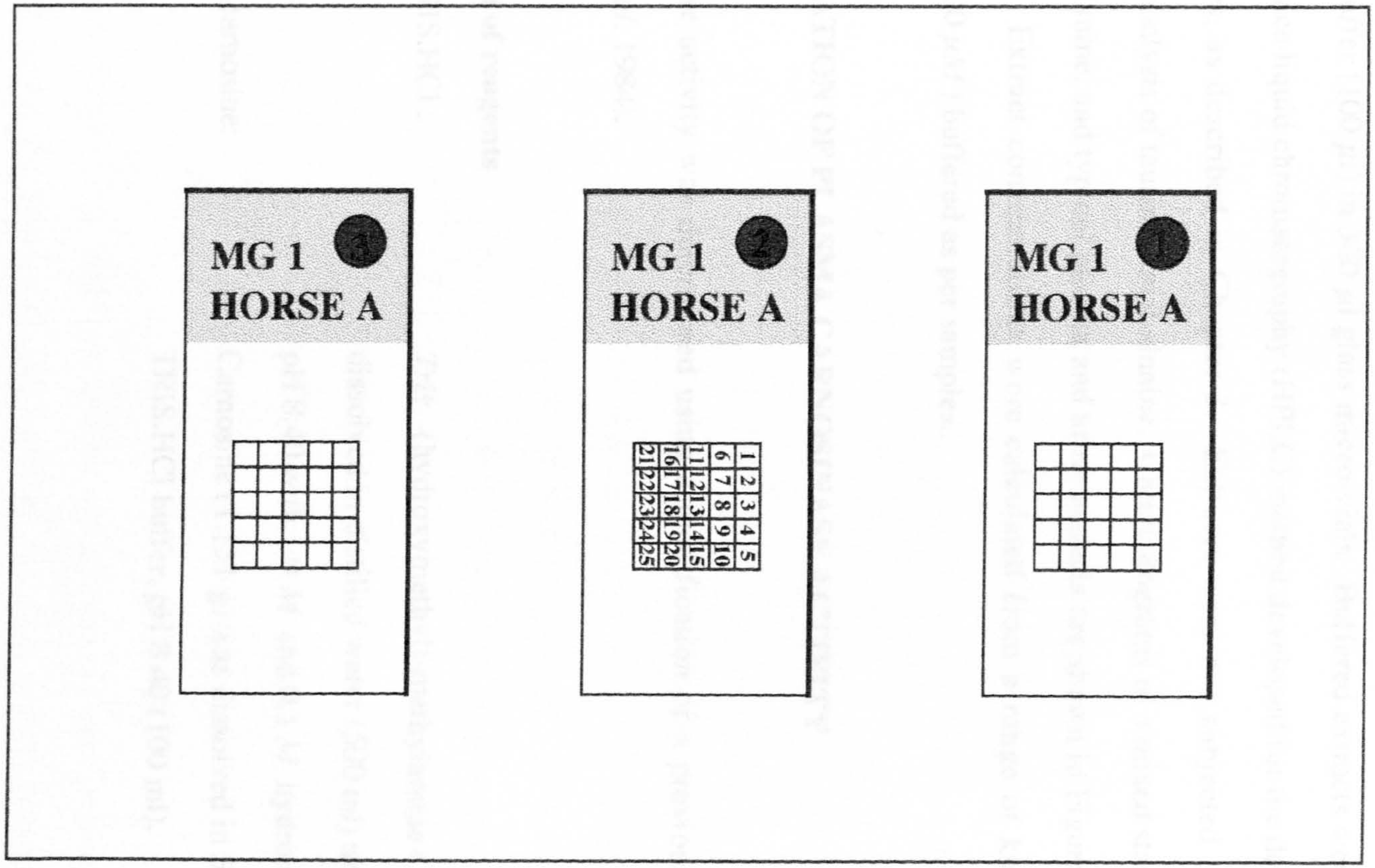

DORSAL
CAUDAL
CRANIAL
VENTRAL
1
2
3
4
5
6
7
8
9

Figure 2.2 Individual muscle fibre mounting procedure.


MG 1
HORSE A
3
MG 1
HORSE A
2
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20
21 22 23 24 25
MG 1
HORSE A
1

2.5 DETERMINATION OF ß-ALANINE AND TAURINE CONCENTRATIONS IN PLASMA, URINE AND MUSCLE

Neutralized perchloric acid extracts of muscle and sulphosalicylic acid extracts of plasma were prepared as described in Section 2.2. Extracts (20 μl) were buffered to pH 9.65 with aqueous 400 m*M* borate buffer (100 μl) in 300 μl glass micro-vials. Buffered extracts were analysed by the high-performance liquid chromatography (HPLC) method developed for the determination of muscle imidazoles, as described in Chapter 3. Extracts were not subjected to solid-phase extraction for the analysis of taurine and ß-alanine. Chromatograms of a mixed standard solution of taurine and ß-alanine, and typical plasma and urine extracts are shown in Figures 2.3, 2.4 and 2.5, respectively. Extract concentrations were calculated from a range of known external standards (10 - 1000 μM) buffered as per samples.

2.6 DETERMINATION OF PLASMA CARNOSINASE ACTIVITY

Plasma carnosinase activity was determined using a modification of a previously published method (Bando *et al.* 1984).

2.6.1 Preparation of reagents

Buffer, 50 m*M* TRIS.HCl : *Tris* -(hydroxymethyl)-methylamine (3.028 g) was dissolved in distilled water (500 ml) and titrated to pH 8.40 with 1.0 *M* and 0.1 *M* hydrochloric acid.

Substrate, 50 m*M* carnosine: Carnosine (1.131 g) was dissolved in 50 m*M* TRIS.HCl buffer, pH 8.40 (100 ml).

2.6.2 Assay

Plasma (50 μl) was diluted with aqueous 50 m*M* TRIS.HCl buffer, pH 8.40 (150 μl), vortex-mixed and pre-incubated for 5 min at 37°C. Aqueous 50 m*M* carnosine (50 μl) was added. The solution was vortex-mixed briefly and incubated for 120 min at 37°C. The reaction was stopped

and protein precipitated by the addition of ice-cold perchloric acid (750 μl). The extract was vortex-mixed, cooled on ice for 5 min and subsequently centrifuged for 5 min at 12000 g at ambient temperature. The supernatant fluid (500 μl) was neutralized with aqueous 2.1 *M* potassium hydrogen carbonate (200 μl). The neutralized extract was mixed, cooled on ice and centrifuged as described previously. The supernatant fluid was collected and analysed for histidine concentration by HPLC. A linear relationship between incubation time and histidine produced was evident for incubation times up to 120 min. The plasma carnosinase assay was established and validated using human plasma

2.7 DETERMINATION OF TISSUE CARNOSINASE ACTIVITY

Tissue carnosinase activity was determined by a modification of a previously published method (Lenney 1990).

2.7.1 Preparation of reagents

Buffer, 50 m*M* TRIS.HCl:	*Tris* -(hydroxymethyl)-methylamine (3.028 g) was dissolved in distilled water (500 ml) and titrated to pH 8.8 with 1.0 *M* and 0.1 *M* hydrochloric acid.
Substrate, 100 m*M* carnosine:	Carnosine (2.262 g) was dissolved in 50 m*M* TRIS.HCl buffer, pH 8.8 (100 ml).
Co-factors, 0.5 m*M* Mn^{2+} : and 20 m*M* dithiothreitol	Manganese dichloride tetrahydrate (9.90 mg) and DL-dithiothreitol (0.308g) were dissolved in distilled water (100 ml).

2.7.2 Assay

Freeze-dried powdered tissue (5 mg) was homogenized (Ultra-Turax motorized homogenizer) for 15 sec in ice-cold 50 m*M* TRIS.HCl buffer, pH 8.8 (700 μl). Further TRIS.HCl buffer (100 μl) or co-factor solution containing 0.5 m*M* Mn^{2+} and 20 m*M* dithiothreitol was added. The homogenate was vortex-mixed briefly and pre-incubated for 5 min at 37°C. Aqueous 100 m*M* carnosine (200 μl) was added, and the homogenate was vortex-mixed and incubated for 120 min

at 37°C. The reaction was stopped by the addition of ice-cold 0.5 *M* perchloric acid (1000 μl). The extract was vortex-mixed, cooled on ice for 5 min and subsequently centrifuged for 5 min at 12000 g at ambient temperature. The supernatant fluid (500 μl) was neutralized with 2.1 *M* potassium hydrogen carbonate (200 μl), mixed, cooled on ice and centrifuged as described previously. The supernatant fluid was collected and analysed for histidine concentration by HPLC. A linear relationship between incubation time and histidine produced was evident for incubation times up to 120 min.

Values for the coefficients of variation for the enzyme assays are given in Table 2.1.

2.8 STATISTICAL METHODS

Unless specified otherwise all data are presented as the mean and standard deviation (mean ± SD). Coefficient of variation (CV) was calculated as follows:

$$CV = (SD \,.\, 100) / \bar{X}$$

Pooled standard deviation (S_p)was calculated according to the following formula:

$$S_p = \sqrt{\Sigma (n_i - 1 \,.\, SD_i^2) / \Sigma n - 1}$$

Where differences in measured parameters between groups were to be compared initial statistical analysis was performed using 1-factor analysis of variance (ANOVA). Where differences were detected significance was determined by the use of a multiple comparison test; Fisher's Protected Least Significant Difference (PLSD). Significance was declared at $p < 0.05$. Simple linear regression analysis where performed was by the least squares method.

Table 2.1 Coefficients of variation for the HPLC analyses and enzyme assays.

Metabolite	Sample	Technique	n	Coefficient of variation (%)	
				Intra-assay	Inter-assay
ß-Alanine	Plasma	HPLC	5	2.0	2.6
	Urine	HPLC	5	1.1	2.4
Taurine	Plasma	HPLC	5	0.9	1.6
	Muscle	HPLC	5	2.5	5.6
Carnosinase	Plasma	Enzyme assay/HPLC	10	5.6	10.6
	Tissue	Enzyme assay/HPLC	10	9.9	10.1

Figure 2.3 Chromatogram of a mixed standard solution containing taurine and ß-alanine at 100 μM.

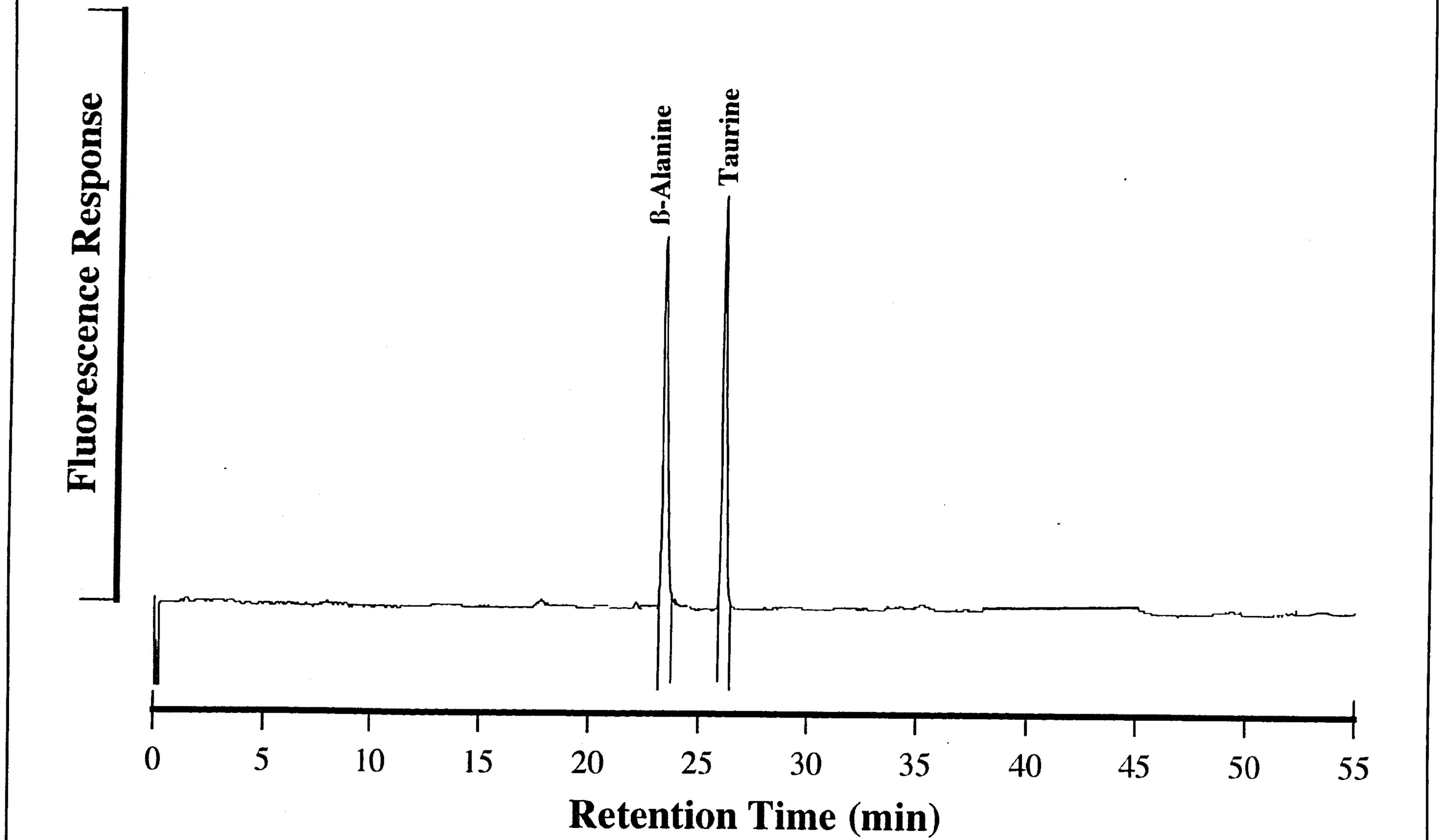

Fluorescence Response
ß-Alanine
Taurine
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

Figure 2.4 Chromatogram of a typical sulphosalicylic acid extract of equine plasma. (* Indicates the position where ß-alanine would appear if it was present in normal equine plasma.)

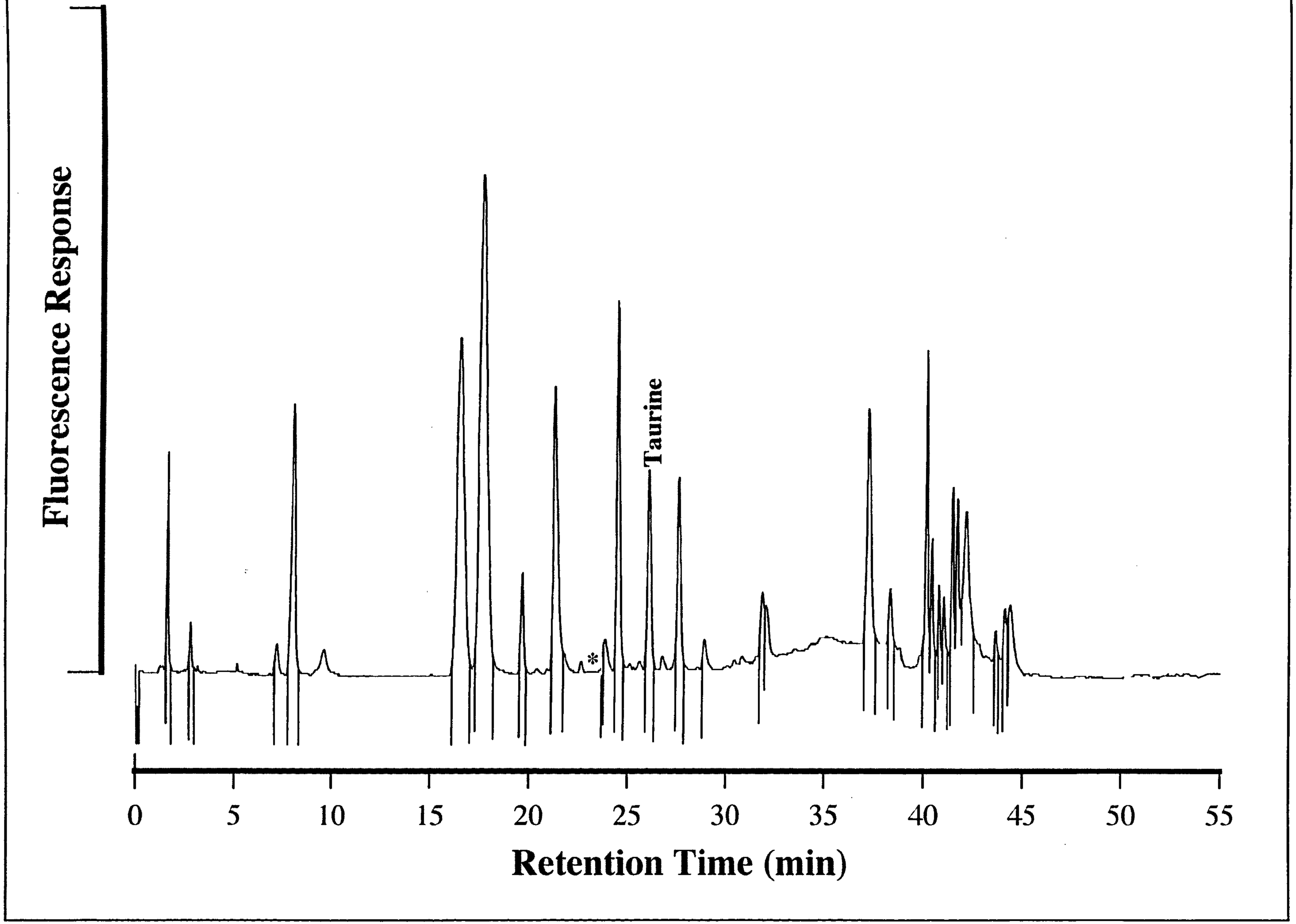
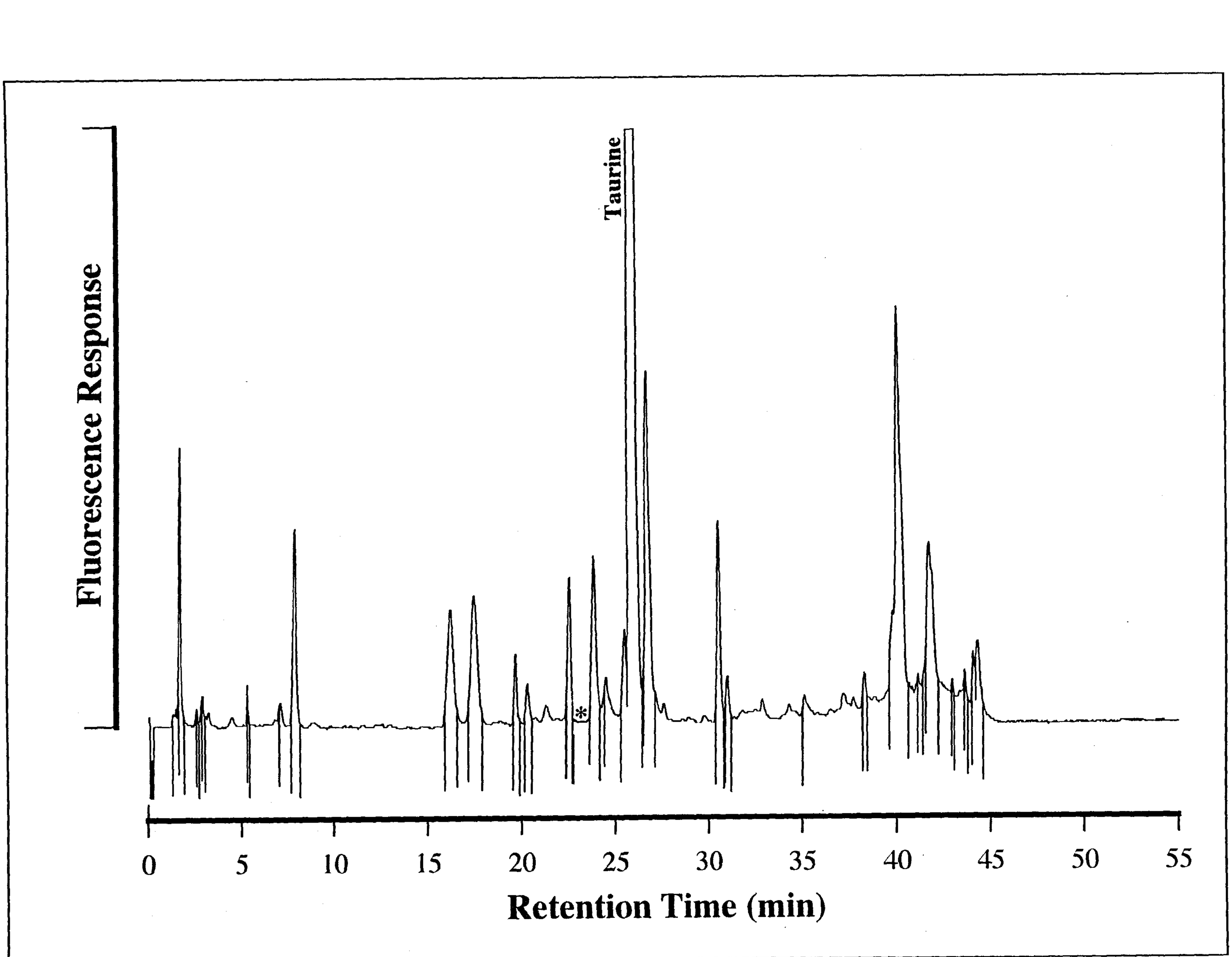

Fluorescence Response
Taurine
*
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

Figure 2.5 Chromatogram of a typical sulphosalicylic acid extract of equine urine. (* Indicates the position where ß-alanine would appear if it was present in normal equine urine.)


Fluorescence Response
Taurine
*
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

2.9 REAGENTS (Ordering information)

Glacial acetic acid 99-101% w/w	Fisons	A/0400/PB17
Acetonitrile 190 (Far UV) super purity solvent	Romil Chemicals	H718
Acetone super purity solvent	Romil Chemicals	H031
N-α-acetyl-L-carnosine	Dr. E. Hultman (gift)	-
ß-Alanine	Sigma	A 7752
Ammonium dihydrogen phosphate	Aldrich	21,600-3
Ammonium sulphide 20% w/w	Aldrich	30,941-9
L-Balenine	Dr. H. Abe (gift)	-
L-Anserine nitrate	Sigma	A 1131
ATP disodium salt: From equine muscle.	Sigma	A 5394
Orthoboric Acid AnalaR	BDH	10058
Calcium chloride dihydrate AnalaR	BDH	10070
L-Carnosine	Sigma	C 9625
Cobalt (II) chloride hexahydrate AnalaR	Sigma	C 2644
Diammonium hydrogen phosphate	Aldrich	33,879-6
DL-Dithiothreitol	Sigma	D 5545
Glycine	Sigma	G 7126
L-Histidine free base	Sigma	H 8000
L-Homocarnosine	Sigma	H 4885
Hydrochloric acid 34.5% v/v AnalaR	BDH	10125
Manganese(II) chloride tetrahydrate	Aldrich	22,127-9
3-Mercaptopropionic acid	Sigma	M 6750
Methanol (205) super purity solvent	Romil Chemicals	H410
Methanol (215) super purity solvent	Romil Chemicals	H409
L-1-Methylhistidine	Sigma	M 9005
L-3-Methylhistidine	Sigma	M 3879
p -Nitrophenol	Sigma	104-8

Perchloric acid 70% w/w	BDH	10176
Phosphoric acid 85% w/w	Aldrich	21,510-4
Potassium hydrogen carbonate AnalaR	BDH	10206
Potassium hydroxide 87%	Sigma	P 1767
o -phthaldialdehyde reagent solution (incomplete)	Sigma	P 7914
Sodium acetate anhydrous	Sigma	S 5889
Sodium acetate trihydrate	Sigma	S 8625
Sodium hydroxide 97.5%	Sigma	S 0899
Sodium pentanesulphonate	Sigma	P 0299
5-Sulphosalicylic acid	Sigma	S 0640
Taurine	Sigma	T 0625
Tetrahydrofuran super purity solvent	Romil Chemicals	H718
Triethylamine 99+%	Aldrich	23,962-3
Tris-(hydroxymethyl)-methylamine	Sigma	T 1503

CHAPTER 3

DEVELOPMENT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHODS FOR THE ANALYSIS OF CARNOSINE, ITS ANALOGUES, AND THEIR METABOLITES IN EQUINE BODY FLUIDS AND TISSUES.

3.1 GENERAL INTRODUCTION

The quantification of the concentrations of carnosine, its analogues anserine, balenine, homocarnosine and N-α-acetylcarnosine, and their metabolites histidine, 1-methylhistidine, 3-methylhistidine in equine plasma, urine and tissues (skeletal and smooth muscle, diaphragm, myocardium, liver, kidney, lung, spleen, cerebellum and medulla), required the development of selective and sensitive analytical methodologies.

Thin-layer chromatography, electrophoresis, and ion-exchange chromatography have previously been used for the measurement of amino acid and imidazole concentrations in biological fluids and tissues (Efron 1969; Smith 1969; Wadman *et al.* 1971; Wadman and de-Bree 1976). However, limitations associated with these techniques including limited selectivity, poor sensitivity and long analysis times, have resulted in their replacement by high-performance liquid chromatography (HPLC). HPLC has proved to be a highly versatile and adaptable analytical technique for the determination of the concentrations of very many low molecular mass compounds in physiological samples including; lipids, vitamins, steroid hormones, carbohydrates, nucleotides, nucleosides and nucleo-bases, organic acids, catecholamines, imidazoles, amines, amino acids and small peptides. Probably the greatest advantage of HPLC over the more traditional techniques is its capacity to resolve and quantify many, often 30 or more, compounds simultaneously with high accuracy, precision and sensitivity; although the rate of sample throughput may be compromised somewhat.

3.2 DETERMINATION OF CARNOSINE AND OTHER BIOGENIC IMIDAZOLES IN EQUINE PLASMA BY ISOCRATIC REVERSED-PHASE ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Dunnett and Harris (1992).

3.2.1 Introduction

Difficulties were encountered in the adaptation of previously published methods for the measurement of tissue carnosine contents, to the determination of carnosine in equine plasma

owing to the low plasma content and tendency for co-elution with amino acids or other small peptides. Analysis times were typically 30 - 60 min and the complex chromatograms produced contain superfluous information. An HPLC method for the quantification of carnosine and anserine in rabbit serum (Kurisaki and Hiraiwa 1988) proved to be grossly inaccurate when applied to equine plasma. An new analytical method was developed which combined isocratic reversed-phase ion-pair HPLC with sorbent extraction to provide rapid, selective and sensitive detection of carnosine, histidine, anserine, and 1-methylhistidine in plasma with previously unobtained specificity.

3.2.2 Experimental

Instrumentation and reagents

The HPLC system comprised a Constametric 3000 pump (LDC Analytical, Stone, Staffs., UK), a Rheodyne 7125 injector with a 200 μl sample loop (Cotati, CA., USA.), a LC-UV variable wavelength ultra-violet spectrophotometric detector (Unicam, Cambridge, UK) and a LKB 6500 flat-bed potentiometric recorder (LKB Biochrom, South Croydon, UK). A Sepralyte ODS 40 μm sacrificial column (50 x 4.6 mm I.D.) (Analytichem International, Harbor City, CA., USA.) and a Rheodyne in-line filter (0.5 μm pore size) (Cotati, CA, USA.) were inserted between the pump and the injector. Ultra-violet absorption spectra were recorded on a Unicam SP800 ultra-violet spectrophotometer (Unicam, Cambridge, UK.).

All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).

Sample deproteinization and solid-phase extraction

Heparinized plasma (1ml) was deproteinized with 200 μl 30% (w/v) 5-sulphosalicylic acid (SSA), and centrifuged. Plasma SSA extracts (500 μl) were loaded onto 100 mg / 1 ml Bond Elut PRS (propylsulphonyl) solid-phase extraction (SPE) cartridges (International Sorbent Technology, Hengoed, UK) previously conditioned with methanol (4 ml) and 1.0 *M* phosphoric

acid (4 ml). Interfering substances were washed from the cartridges with 1.0 *M* phosphoric acid (2 x 500 μl) and the eluate discarded. The sorbent was air dried and the isolates (carnosine and other imidazoles) were eluted with HPLC mobile phase (2 x 500 μl). Solvents at all stages were drawn through the cartridges at a flow-rate of 1 ml min^{-1}. A 200 μl aliquot was injected onto the HPLC column.

Chromatography

Chromatography was performed on a Hypersil ODS 3 μm (150 x 4.6 mm I.D.) analytical column (Shandon Scientific, Runcorn, UK.) protected by a Hypersil ODS 5 μm (20 x 4.6 mm I.D.) guard column.

The compounds of interest were eluted isocratically at ambient temperature using a mobile phase comprising an aqueous solution of 200 m*M* ammonium dihydrogen phosphate $(NH_4)H_2PO_4$ and 100 m*M* sodium pentanesulphonate adjusted to pH 2.0 with concentrated phosphoric acid, and containing 4% (v/v) acetonitrile. The mobile phase was filtered through 0.45 μm (HVLP 047 PTFE filters, Millipore UK Ltd., Watford, Herts., UK.) and degassed by helium sparging prior to use and periodically throughout the day. The mobile phase was freshly prepared each day. The flow-rate was 0.8 ml min^{-1}. UV absorption was measured at 220 nm with the detector sensitivity set for full-scale deflection (FSD) at 320 milliabsorbance units (mAU). Plasma concentrations were determined by comparing sample peak heights to those of external standards.

Standard preparation

Individual 10 m*M* stock standard solutions of carnosine, anserine, histidine and 1-methylhistidine were prepared by dissolving the required weight of each compound in HPLC grade water (10 ml). Working standard solutions for each compound were prepared over the concentration range 5 - 30 μM by dilution of stock standards with mobile phase. Both stock standard solutions and working standard solutions were stored at -20^{0}C when not used.

Recovery study

Pooled equine plasma was spiked at 25 μM with a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine. Overall extraction recoveries (n = 5) for the combined deproteinization and sorbent extraction stages were determined by comparing the chromatograms obtained from the spiked plasma extracts with those obtained from a mixed standard solution containing 25 μM 1-methylhistidine, histidine, anserine and carnosine. The concentration of these compounds in the plasma prior to spiking was determined and taken into account when calculating overall recoveries.

Reproducibility study

Standards for the determination of the precision and accuracy of the analysis were prepared from pooled equine plasma extract which was spiked at 25 μM with 1-methylhistidine, histidine, anserine and carnosine. Repeated injections of the spiked extract (n = 5) were made on day 1, day 5 and day 10 to assess the inter-assay variation. Intra-assay variation was determined by repeated injections (n = 5) of the spiked extract on three occasions within day 1.

Lower detection limits

The lower limits of detection for the compounds of interest were determined by injecting individual standards of 1-methylhistidine, histidine, anserine and carnosine sufficient to elicit FSD on the recorder at a detector sensitivity setting of 10 mAU. The height of the smallest detectable peak was 5mm which was equivalent to a signal : noise ratio of 2:1.

3.2.3 Results and discussion

Chromatography

Spectra over the UV range 190 - 450 nm were recorded for the imidazole compounds of interest. Their mean λ_{max} values occurred at a wavelength of 220 ± 5 nm (carnosine, λ_{max} = 223 nm) in 0.1M $(NH_4)H_2PO_4$ (pH 2.0). The reference cell contained 0.1M $(NH_4)H_2PO_4$. The UV spectra of several other amino acids were also recorded and exhibited λ_{max} values at slightly lower wavelengths (ß-alanine, λ_{max} = 205 nm). At 220 nm the absorbance ratio of ß-alanine :

carnosine was 1.00 : 5.88 for equimolar solutions. A detection wavelength of 220 nm was selected.

Inevitably there is the potential for significant interference when utilizing UV detection at such a non-specific wavelength. In addition to the amino acids, many other polar low molecular mass compounds ($M_r < 2000$), endogenous to plasma, display strong UV absorption at 220 nm. Some such compounds are; creatine, creatinine, purine nucleotides, nucleosides and bases, and carboxylates (lactate and pyruvate). However, it has been demonstrated previously that for a non-polar stationary phase and a polar mobile phase, retention of the biogenic imidazoles is maximised at low pH (O'Dowd *et al.* 1988), and furthermore that creatine, creatinine and the purine based compounds are minimally retained (Sellevold *et al.* 1986).

A variety of columns and mobile phase parameters were evaluated in order to optimize the resolution of the various imidazole compounds and to further minimize any potential interference. Aqueous potassium, or sodium, dihydrogen phosphate buffers have generally formed the basis of previous eluents (O'Dowd *et al.* 1988) for the HPLC of carnosine and the other imidazoles (O'Dowd *et al.* 1988; Kurisaki and Hiraiwa 1988). The use of alkali metal phosphates during the present method development caused considerable peak tailing of these basic compounds and was apparent in a previous method (O'Dowd *et al.* 1988). The alternative use of $(NH_4)H_2PO_4$ resulted in a marked improvement in peak symmetry. The effect of a range of ion-pairing agents (sodium pentanesulphonate, sodium heptanesulphonate, sodium octanesulphonate and sodium dodecyl sulphate) and acetonitrile as the organic modifier, on the retention and resolution of the compounds of interest was investigated. Sodium pentanesulphonate (100 mM) and 4% acetonitrile produced the best resolution within the optimum capacity factor (K') range , $1 < K' > 10$. Of the different analytical columns evaluated; Apex ODS1 5 μm, Apex Phenyl 5 μm, Spherisorb ODS2 5 μm, Hypersil ODS 5 μm and Hypersil ODS 3 μm, superior resolution was obtained with the latter. The resultant chromatography conditions produced a good separation of 1-methylhistidine, histidine, anserine and carnosine as shown in Figure 3.1. The retention times for 1-methylhistidine, histidine,

anserine and carnosine were 4.6, 5.8, 7.4 and 9.2 min, respectively. The efficiency of the separation is indicated by a resolution factor, $R_S > 4.2$ for all adjacent peaks.

Sample deproteinization and solid-phase extraction

Deproteinization of plasma using traditional reagents such as 1.0 M perchloric acid, 0.1% trifluoroacetic acid or methanol (3 - 4 equivalent volumes) produce an unwanted dilution. This is particularly undesirable in the analysis of carnosine where the plasma concentration is known to be generally of the order of 10 μM. The addition of a small volume (100 μl) of a concentrated solution of SSA efficiently precipitates plasma protein, minimises the dilution effect and is more reproducible than adding SSA in solid form. Chromatography of SSA deproteinized plasma was inadequate for the determination of carnosine as the carnosine peak eluted within the tailing side of a large front peak, thus making quantification impossible at low levels. In addition, the peaks arising from 1-methylhistidine, histidine and anserine were entirely obscured by this large front peak. Furthermore, large peaks evolving from well retained compounds continued to appear in the chromatogram up to 22 min after sample injection and only after this time did the base line absorbance begin a significant reduction towards the pre-injection value. SPE was employed to provide pre-chromatography sample clean-up. A variety of methodologies were investigated to determine the effectiveness of the technique. At low pH values, carnosine (and the other imidazole molecules) exist as cationic species. The degree of ionization of the imidazole ring and the terminal amine group approaches 100% at pH 2.0. This property was exploited by use of a cation-exchange mechanism for sorbent extraction. The requirement for an acid environment throughout the extraction procedure was simplified by the inherent acidic nature of the SSA deproteinized plasma and by the requirement for an acidic final extract to provide good chromatography in the pH 2.0 mobile phase. Using the SPE method described, an excellent purification of the deproteinized plasma was achieved, and few, if any, interference peaks were present in the final extract. The contrast in sample quality between pre- and post-SPE is demonstrated for a typical equine plasma extract in Figure 3.2.

Figure 3.1 HPLC separation of a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine.

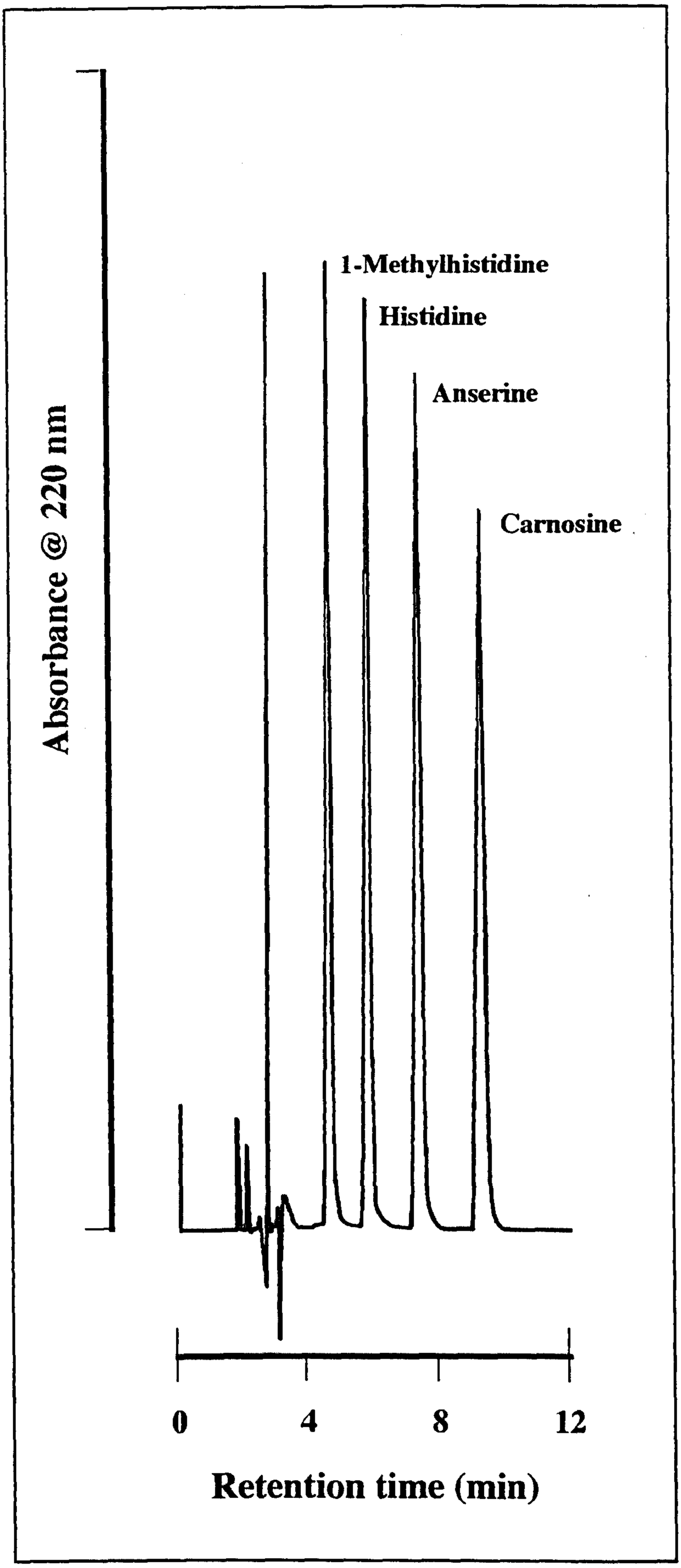
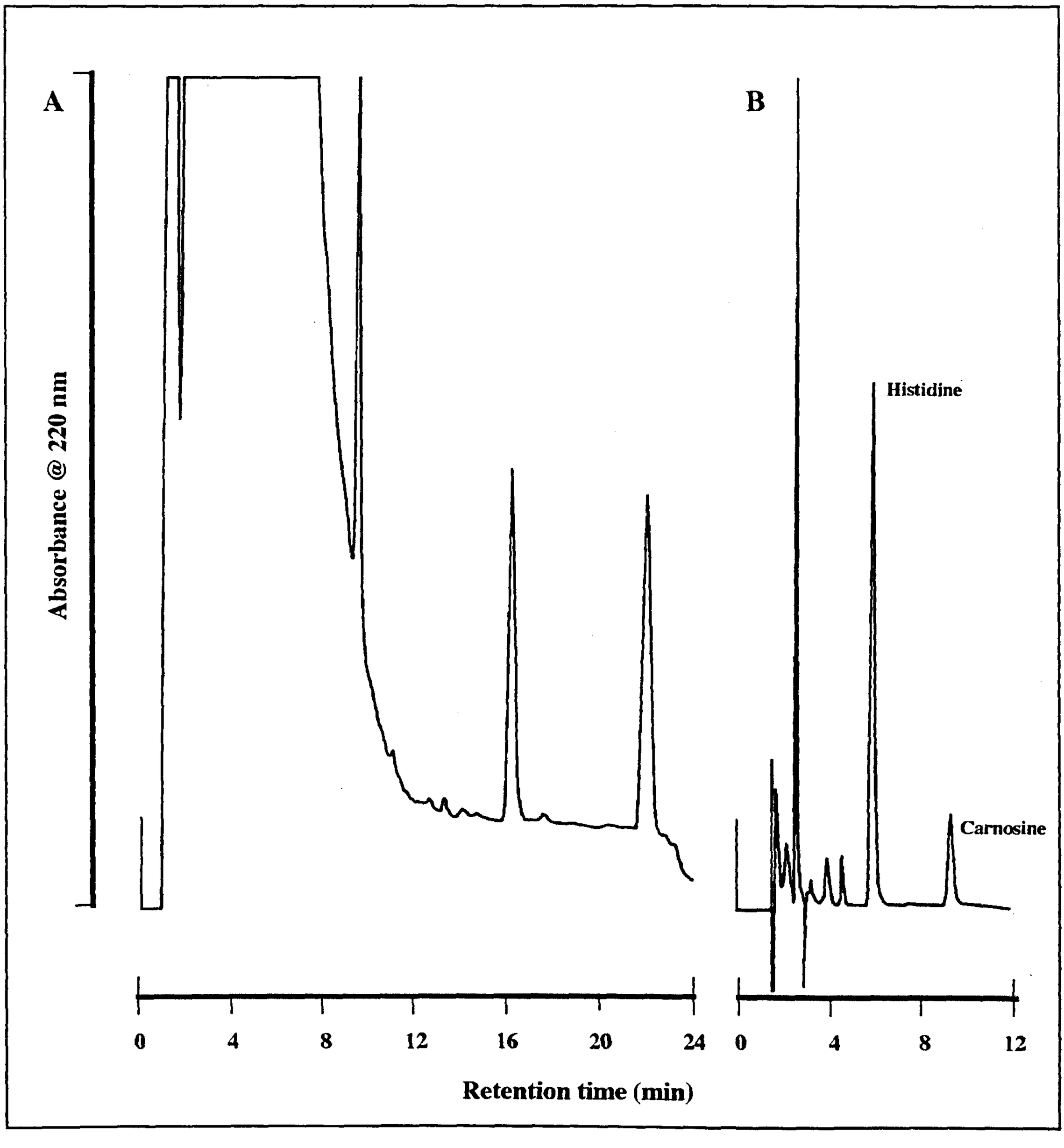

1-Methylhistidine
Histidine
Anserine
Carnosine
Absorbance @ 220 nm
0
4
8
12
Retention time (min)

Figure 3.2 HPLC separation of a SSA extract of equine plasma: A) Pre-SPE. B) Post-SPE.


A
B
Absorbance @ 220 nm
Histidine
Carnosine
0
4
8
12
16
20
24
0
4
8
12
Retention time (min)

Spiking of a final equine plasma extract with a mixed standard, prepared from authentic compounds, produced only single peaks for putative carnosine and histidine endogenous to plasma , as shown in Figure 3.3. Peaks for anserine and 1-methylhistidine in the spiked extract were absent in the normal final extract. Spiked canine plasma extracts displayed single peaks for each of the imidazoles.

Standards

Standard curves for carnosine and the other imidazoles showed a linear relationship between peak height and concentration in the range 5 - 30 μM. The linear regression equations for each compound were 1-methylhistidine, $y = 6.21x + 0.61$ ($r > 0.99$); histidine, $y = 5.91x + 0.14$ ($r > 0.99$); anserine, $y = 5.48x + 0.54$ ($r > 0.99$); carnosine, $y = 4.65x + 0.11$ ($r > 0.99$) where y = peak height (mm) and x = concentration (μM).

Recovery and reproducibility studies

Overall recoveries (mean ± CV, %) from spiked plasma were 1-methylhistidine, 103.7 ± 9.4 %; histidine, 105.4 ± 9.9 %; anserine, 97.0 ± 4.4 %; carnosine, 101.8 ± 9.4 %. This deproteinization and extraction technique provided a highly selective determination of the biogenic plasma imidazoles.

Values for typical intra-assay and inter-assay variation are given in Tables 3.1 and 3.2, respectively. In these tables, imidazole concentrations above 25 μM are a consequence of the endogenous plasma content. The mean intra-assay coefficients of variation (CV) were 7.9% or less for the compounds of interest in spiked plasma. Inter-assay mean CV were 6.4% or less for the compounds of interest.

Lower detection Limits

Lower detection limits for 1-methylhistidine, histidine, anserine and carnosine were 58.3, 65.9, 71.5 and 80.1 nM , respectively.

Figure 3.3. HPLC separation of a SSA extract of plasma spiked with a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine.

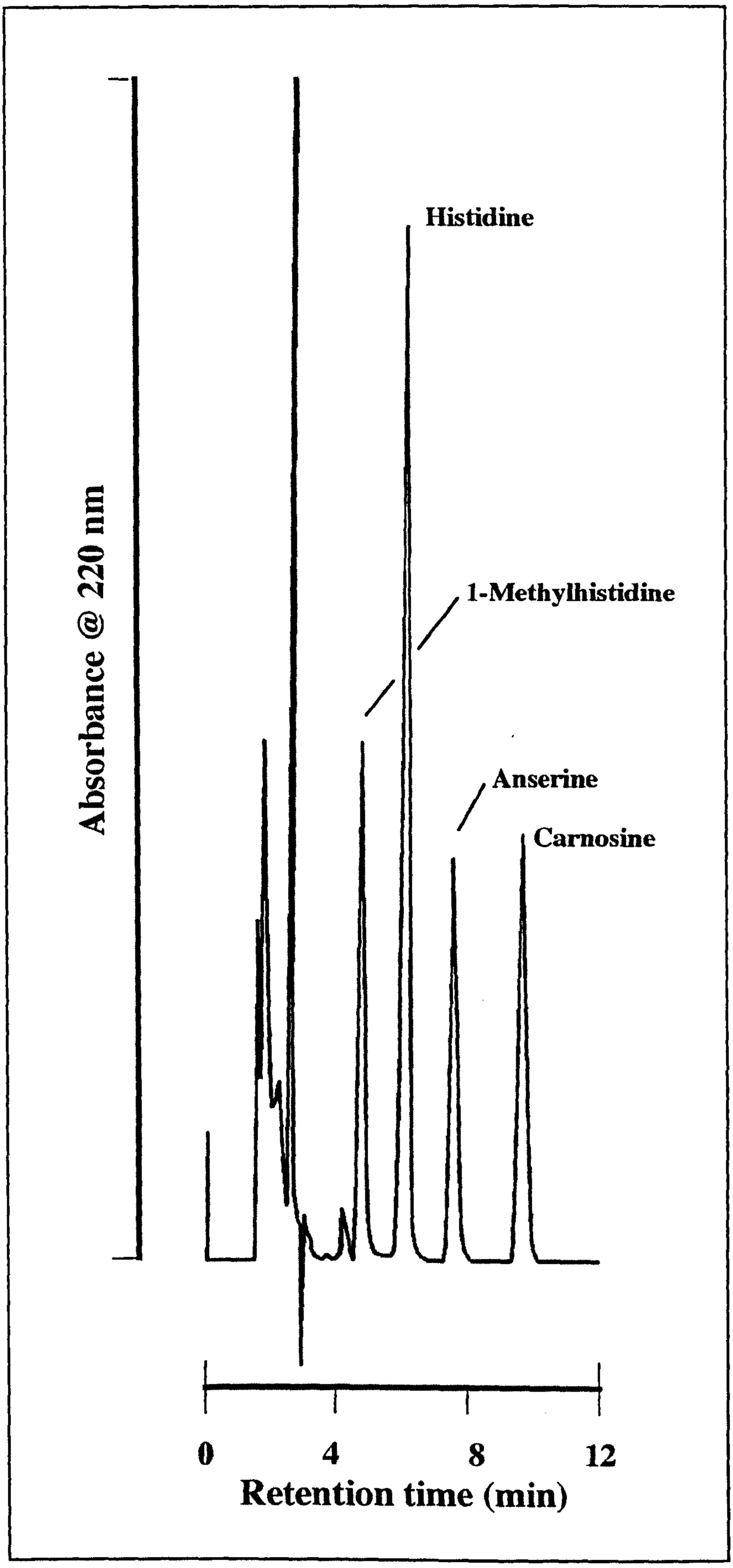

Histidine
1-Methylhistidine
Anserine
Carnosine
Absorbance @ 220 nm
0
4
8
12
Retention time (min)

Table 3.1 Intra-assay precision and accuracy for the determination of the biogenic imidazoles in equine plasma.

Session	1-Methylhistidine		Histidine		Anserine		Carnosine	
	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)
1	29.11 ± 1.80	6.2	67.96 ± 1.29	1.9	23.06 ± 1.88	8.2	31.96 ± 1.05	3.3
2	31.10 ± 1.49	4.8	71.49 ± 1.34	1.9	22.48 ± 1.65	7.3	33.29 ± 1.02	3.1
3	29.96 ± 1.70	5.7	70.42 ± 1.64	2.3	22.90 ± 1.84	8.0	32.50 ± 0.94	2.9
Mean	30.06 ± 1.67†	5.6	69.96 ± 1.43†	2.0	22.81 ± 1.79†	7.9	32.58 ± 1.00†	3.1

† = Pooled intra-assay SD.

Table 3.2 Inter-assay precision and accuracy for the determination of the biogenic imidazoles in equine plasma.

Day	1-Methylhistidine		Histidine		Anserine		Carnosine	
	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)	Concentration (μM)	CV (%)
Day 1	29.33 ± 2.41	8.2	74.43 ± 2.63	3.5	24.25 ± 1.09	4.5	31.16 ± 1.57	5.0
Day 5	31.12 ± 1.47	4.7	71.49 ± 1.34	1.9	22.48 ± 1.65	7.3	33.11 ± 1.35	4.1
Day 10	30.93 ± 1.83	5.3	66.95 ± 5.79	8.7	24.01 ± 1.45	6.0	29.47 ± 1.13	3.8
Mean	30.46 ± 1.94†	6.4	70.96 ± 3.75†	5.3	23.61 ± 1.42†	6.0	31.25 ± 1.36†	4.4

† = Pooled inter-assay SD.

Equine plasma extracts were also injected and analysed at 254 nm and 280 nm, however, no peaks were present in the chromatograms. No detectable peaks at these two wavelengths is clear evidence for the absence of interfering compounds such as; purine nucleotides, nucleosides and bases, tyrosine, phenylalanine, tryptophan and their metabolites, and other aromatic species.

The HPLC method developed provided a rapid, selective, sensitive and reproducible analysis of carnosine, histidine, anserine and 1-methylhistidine in plasma.

3.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IMIDAZOLE DIPEPTIDES, HISTIDINE, 1-METHYLHISTIDINE AND 3-METHYLHISTIDINE IN EQUINE TISSUES AND INDIVIDUAL MUSCLE FIBRES

Dunnett and Harris (1995b)

3.3.1 Introduction

Several HPLC methods have been developed to determine imidazole compounds in tissue samples. The techniques employed include ion-exchange elution with UV detection (Abe and Ohmama 1987) or with derivatization and fluorescence detection (Carnegie *et al.* 1983), isocratic reversed-phase elution with UV detection (O'Dowd *et al.* 1988; O'Dowd *et al.* 1992), iso-cratic reversed-phase elution with pre-column derivatization and fluorescence detection (Kasziba *et al.* 1988), combined reversed-phase and ion-exchange gradient elution with pre-column derivatization and fluorescence detection (Teahon and Rideout 1992), and reversed-phase gradient elution with pre-column derivatization and fluorescence detection (Wideman *et al.* 1978; Godel *et al.* 1984; Qureshi *et al.* 1984; Boon *et al.* 1989).

Since reversed-phase HPLC of *o* -phthaldialdehyde / 2-mercaptoethanol pre-column derivatized amino acids was introduced (Lindroth and Mopper 1979), various modifications have been incorporated into the technique. Initially the only imidazole measured was histidine (Jones *et al.* 1981; Godel *et al.* 1984), although subsequent alterations made possible the additional measurement of 1-methylhistidine and 3-methylhistidine (Jones and Gilligan 1983). Later

modifications to the separation parameters and the derivatization chemistry (substituting 3-mercaptopropionic acid for 2-mercaptoethanol) enabled the resolution of histidine, 1-methylhistidine and 3-methylhistidine and carnosine in extracts of plasma, tissues and urine (Qureshi *et al.* 1984; Jones and Gilligan 1983; Qureshi *et al.* 1986; Furst *et al.* 1990). These methods, however, were not developed for the specific analysis of imidazole-derived amino acids and small peptides. In an attempt to provide specificity in the analysis of carnosine, anserine and 3-methylhistidine, reversed-phase HPLC of *o*-phthaldialdehyde / 3-mercaptopropionic acid (OPT) derivatives has been adapted by incorporating column-switching (Teahon and Rideout 1992).

None of the previous methods encompassed all of the imidazoles. A new method was developed which combined solid-phase extraction, reversed-phase HPLC with binary gradient elution, automated pre-column derivatization with *o*-phthaldialdehyde / 3-mercaptopropionic acid, and fluorescence detection to enable the selective determination of carnosine, anserine, balenine, homocarnosine, histidine, 1-methylhistidine and 3-methylhistidine in tissue extracts within a single chromatographic run. In conjunction with a micro-extraction technique the method allowed the quantification of the compounds of interest in individual skeletal muscle fibres, where sample weights can be as low as 0.8 μg.

3.3.2 Experimental

Instrumentation and reagents

The HPLC system comprised two Constametric 3000 pumps (LDC Analytical, Stone, Staffs., UK.), a LDC high pressure solvent mixer, a Waters WISP 712 autosampler with auto-addition facility (Waters Chromatography, Watford, Herts., UK.), a Spectrovision FD 300 dual monochromator fluorescence detector (Severn Analytical Ltd., Shefford, Beds., UK.), a LDC MP 3000 chromatography workstation - gradient controller, and a LDC printer. A Sepralyte ODS pellicular 40 μm sacrificial column (20 x 2.1 mm I.D.) (Analytichem International, Harbor City, CA., USA.) and a Rheodyne 0.5 μm in-line filter (Cotati, CA., USA.) were connected between the high pressure mixer and the autosampler.

All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).

Tissue deproteinization and solid-phase extraction

Powdered freeze-dried tissue (10.00 ± 2.00 mg) was extracted in 0.5 *M* perchloric acid (100 μl / mg tissue) on ice for 15 min with regular vortex mixing. The extraction mixture was centrifuged at 12,000 g for 5 min at ambient temperature. Tissue perchloric acid extracts (500 μl) were loaded onto 100 mg / 1 ml Isolute PRS (propylsulphonyl) SPE columns previously conditioned with methanol (4 ml) followed by 1.0 *M* phosphoric acid (4 ml). Interfering compounds were washed from the columns with 1.0 *M* phosphoric acid (2 x 500 μl), water (500 μl) and 0.4 *M* disodium tetraborate buffer, pH 9.65 with sodium hydroxide (250 μl). The sorbent was dried under vacuum for two minutes. The isolates were eluted with 0.4 *M* disodium tetraborate buffer, pH 9.65 (3 x 250 μl). A flow-rate of 2 ml min^{-1} was used at all stages.

Individual muscle fibre extraction

Individual muscle fibres were extracted with 75% methanol / 25% 0.4 *M* disodium tetraborate buffer pH 9.65 (100 μl). Extractions were performed on ice with regular vortex mixing for 15 min whilst ensuring that the muscle fibres were permanently submerged. Extracts were frozen in liquid nitrogen, freeze-dried, re-dissolved in 0.4 *M* disodium tetraborate buffer pH 9.65 (50 μl) and stored at -85°C until analysis.

Chromatography

Chromatography was performed on a Hypersil ODS (3 μm, 150 x 4.6 mm I.D.) analytical column protected by a Hypersil ODS (5 μm, 20 x 4.6 mm I.D.) guard column, at ambient temperature, utilizing a binary gradient formed from Solvent A; 12.5 m*M* sodium acetate, pH 7.2 (995 ml) and tetrahydrofuran (5 ml), and Solvent B; 12.5 m*M* sodium acetate, pH 7.2 (500 ml), methanol (350 ml) and acetonitrile (150 ml). The 12.5 m*M* pH 7.2 acetate buffer was prepared by mixing isomolar solutions of sodium acetate and acetic acid. The solvents were

filtered to 0.45 μm and degassed by helium sparging prior to and throughout the analytical run. The mobile phase was freshly prepared for each new sample batch. The composition of the gradient was: 0 - 3 min., 0% solvent B; 20 min., 35% B; 36 min., 60% B; 40 min., 100% B; 43 min., 100% B; 45 - 55 min., 0% B. The flow-rate was 1.0 ml min^{-1} and chromatographic run-time was 55 min. per sample. The excitation wavelength of the detector was 330 nm and the emission wavelength was 450 nm. The derivatization reagent was prepared by mixing 3-mercaptopropionic acid (80 μl) with *o* -phthaldialdehyde reagent solution (4 ml). The derivatization reagent was stored in the dark at 2°C for 24 hours before use. During the automated derivatization, 25 μl of the extract was mixed with 25 μl of reagent. The reaction proceeded for 90 s before injection onto the column. Fresh reagent was used with each new sample batch. Tissue concentrations were calculated by comparison of the integrated peak areas with those from a range of external standards.

Standard preparation

A mixed stock standard (10 m*M*) solution containing histidine, 1-methylhistidine, 3-methylhistidine, anserine, carnosine, balenine and homocarnosine was prepared by dissolving the required weight of each compound in HPLC grade water (10 ml). Working standard solutions were prepared over the concentration range 0.010 - 1.000 m*M* by dilution of the stock standard with borate buffer. The stock standard solution was stored at -20°C when not used.

Recovery study

Aliquots of pooled perchloric acid extracts of freeze-dried powdered diaphragm muscle were spiked with a mixed standard containing imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine. These samples were spiked to simulate tissue concentrations of 100 mmol kg^{-1} DW (n = 5), 50 mmol kg^{-1} DW (n = 5), 5 mmol kg^{-1} DW (n = 5) and 1 mmol kg^{-1} DW (n = 5). Recoveries following SPE were calculated by comparison with equivalent standards. The concentrations of the endogenous compounds prior to spiking were determined and subtracted when calculating the recoveries.

Reproducibility study

A perchloric acid extract of pooled diaphragm muscle was spiked with a mixed standard of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine to simulate individual muscle concentrations of 100 mmol kg^{-1} DW. The spiked extract was divided into 25 aliquots. Intra-assay variation of the combined SPE and HPLC analysis was determined by repeated injections (n = 5) of the SPE eluates on three occasions within day 1. Inter-assay variation was determined by repeated injections (n = 5) of the SPE eluates on day 1, day 5 and day 10.

Lower detection limits

The lowest levels of detection for the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine were quantified by injecting of 100 μl of a mixed standard which resulted in FSD at a detector sensitivity setting of 20 nA. The minimum area reliably measurable was 3000 area units which approximated to a 5 mm peak at a signal to noise ratio of 3:1. The concentration of the minimum peak was interpolated from the mixed standard.

3.3.3 Results and discussion

Chromatography

Use of pre-column derivatization with OPT for the HPLC analysis of amino acids and primary amines in physiological samples can produce detailed chromatograms containing as many as 30 - 40 peaks (Turnell and Cooper 1982). Such complexity may be advantageous, for example in the clinical diagnosis of aminoacidaemias (Moretti *et al.* 1990) and muscle amino acid profiles during uraemia (Qureshi *et al.* 1989), however, poor resolution between certain amino acid pairs makes it difficult to accurately quantify the area of either peak.

Previously published chromatographic methods applied to the determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in equine tissue extracts failed to resolve all the compounds of interest. Various combinations of columns (Apex Phenyl 5 μm, Apex ODS 5 μm, Spherisorb ODS 2 5 μm and 3 μm, Hypersil ODS 3 μm, Hypersil ODS HC 3 μm and Primesphere ODS 3 μm) and mobile phases (10 m*M*, 12.5 m*M* and 25 m*M* acetate,

phosphate and mixed acetate / phosphate buffers) were evaluated to optimize the resolution of the imidazole compounds from interfering amino acids. An acceptable resolution of the seven imidazole standards alone could not be achieved using the 5 μm packed columns. The optimal separation of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine from 22 amino acids (including taurine, ß-alanine and GABA) was achieved using the Hypersil ODS 3 μm column in conjunction with the 12.5 m*M* acetate buffer binary gradient. As shown in Figure 3.4, the final chromatographic parameters resulted in a good separation of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine, although base-line separation was not quite achieved between 1-methylhistidine and 3-methylhistidine. Retention times for the individual compounds were: histidine, 20.19 min. ; 1-methylhistidine, 21.82 min. ; 3-methylhistidine, 22.23 min. ; anserine, 26.43 min. ; carnosine, 27.45 min. ; balenine, 30.08 min. ; homocarnosine, 31.71 min.

Solid-phase extraction

During the analysis of physiological samples for low levels of the compounds of interest there is the possibility of impaired resolution from citrulline, alanine, taurine and arginine when these amino acids are present at relatively much higher concentrations. SPE employing a benzene propylsulphonyl bonded phase had been used for the extraction of amino acids from urine (Moodie *et al.* 1989), however, this procedure did not discriminate between different classes of amino acids. The presence of the benzene ring in the bonded phase may also induce secondary non-polar interactions thus complicating the adaptation of this method. A propylsulphonyl strong cation-exchange bonded phase was previously shown to be effective in retaining biogenic imidazole compounds from acidic plasma extracts (Dunnett and Harris 1992). SPE was adopted to provide a selective extraction of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine from the other amino acids and small peptides present in tissues. The present SPE method proved highly selective for the imidazole compounds. Most amino acids, including citrulline, alanine and taurine, were absent from the final extract, however arginine (26.01 min.) was fully recovered. Chromatograms of perchloric acid extracts of myocardium pre- and post-SPE are shown in Figure 3.5 and gluteus medius muscle post-SPE in Figure 3.6.

The SPE resulted in an effective concentration of the analytes as separate neutralization and buffering (pH 9.65) of the perchloric acid extracts was avoided. Post SPE extracts of equine tissues were spiked with a mixed standard of the authentic compounds. Subsequent HPLC produced only single peaks at the retention times for the putative compounds.

Standards

The standard curves demonstrated a linear relationship between integrated peak area and concentration in the range 0.010 - 1.000 m*M*. Linear regression equations for each compound were: histidine, y = 65510x + 103 (r = 0.998); 1-methylhistidine, y = 103497x + 1164 (r = 0.997); 3-methylhistidine, y = 100599x + 3682 (r = 0.996); anserine, y = 257649x + 2977 (r = 0.997); carnosine, y = 247476x + 833 (r = 0.997); balenine, y = 211263x + 5845 (r = 0.995), and homocarnosine, y = 294440x + 2597 (r = 0.995). (y = integrated peak area in arbitrary units, x = concentration, m*M*).

Recovery and reproducibility studies

The overall recoveries (mean ± CV, %) of the imidazole compounds from spiked tissue samples following SPE are shown in Table 3.3. Excellent recoveries were obtained for all the compounds of interest. The intra-assay and inter-assay reproducibility (mean ± SD and CV, %) of the combined SPE-HPLC analysis of the compounds of interest are given in Tables 3.4 and 3.5, respectively. The intra-assay mean CV for all the compounds of interest in spiked muscle extracts ranged from 1.2 - 6.3% . The inter-assay mean CV ranged from 1.7 - 2.9% .

Lower detection limits

Lower limits of detectability determined for 100 μl injected at a detector sensitivity setting of 20 nA FSD were 0.010 mmol kg^{-1} DW (1.6 pmoles on column) for histidine, 1-methylhistidine and 3-methylhistidine, and 0.005 mmol kg^{-1} DW (0.8 pmoles on column) for carnosine, anserine, balenine and homocarnosine. Detector sensitivity settings at 10 nA FSD or less resulted in unacceptable signal-to-noise ratios. Injection volumes greater than 100 μl resulted in peak broadening and reduced resolution between 1-methylhistidine and 3-methylhistidine.

Individual fibre extraction validation

Owing to the extremely low sample weights, typically 0.8 - 8.0 μg, it was necessary to minimize the sample extract volume by maximizing detector response. The sample extracts were therefore concentrated by freeze-drying and re-dissolution. As perchloric acid cannot be removed by freeze-drying an alternative solvent for tissue extraction had to be found. Comparative extractions of freeze-dried powdered middle gluteal muscle (1 mg ml^{-1}) between perchloric acid and 3 alternative solvents were performed (n = 4 for each solvent). The relative recoveries of carnosine in three alternative solvents compared to perchloric acid were; 100.9% (75% methanol / 25% 0.4 *M* borate buffer pH 9.65), 87.3% (75% acetonitrile / 25% 0.4 *M* borate buffer pH 9.65) and 101.0% (4% sulphosalicylic acid). Comparative extractions between perchloric acid and 75% methanol / 25% 0.4 *M* borate buffer were made in 12 individual equine muscle fibres. Each fibre was halved; one half was extracted with perchloric acid the other with methanol-borate buffer. The mean (± SD) carnosine in the perchloric acid extracted fibres and the methanol / borate buffer extracted fibres, 46.94 ± 9.66 mmol kg^{-1} DW and 47.21 ± 11.44 mmol kg^{-1} DW were not significantly different ($p > 0.05$).

The present SPE-HPLC method developed and validated for the isolation and quantification of carnosine, anserine, balenine, homocarnosine, histidine, 1-methylhistidine and 3-methylhistidine in equine tissues and individual muscle fibres across a broad concentration range in tissue samples weighing from < 1 μg to 10 mg proved to be selective, sensitive and reproducible. This method avoids the interference from other physiological amino acids and small peptides associated with earlier techniques and for the first time enables the resolution of all seven compounds within a single chromatographic run.

Figure 3.4 HPLC separation of a mixed standard containing the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine.

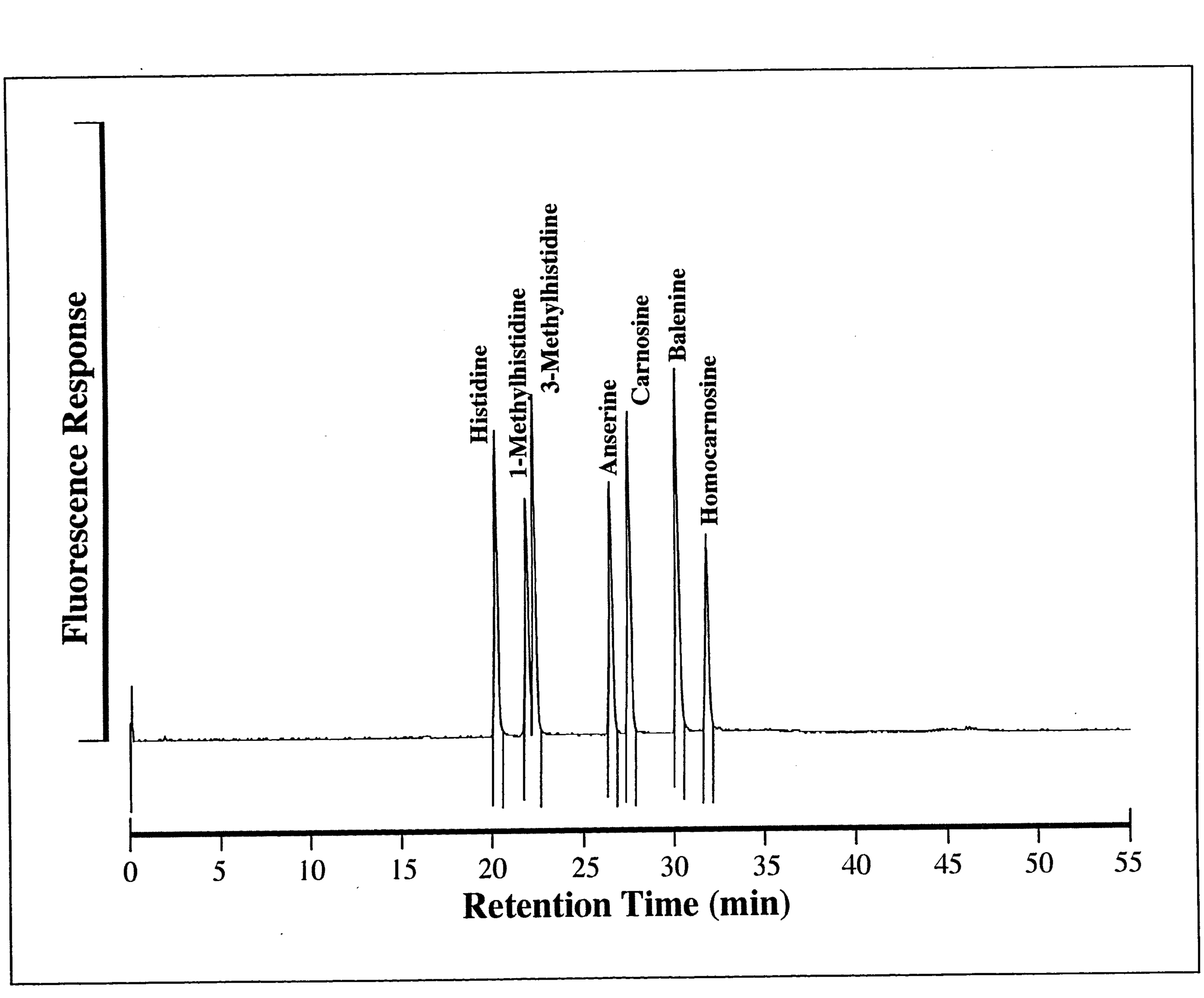

Fluorescence Response
Histidine
1-Methylhistidine
3-Methylhistidine
Anserine
Carnosine
Balenine
Homocarnosine
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

Figure 3.5 HPLC separation of a perchloric acid extract of myocardium: A) Pre-SPE. B) Post-SPE.

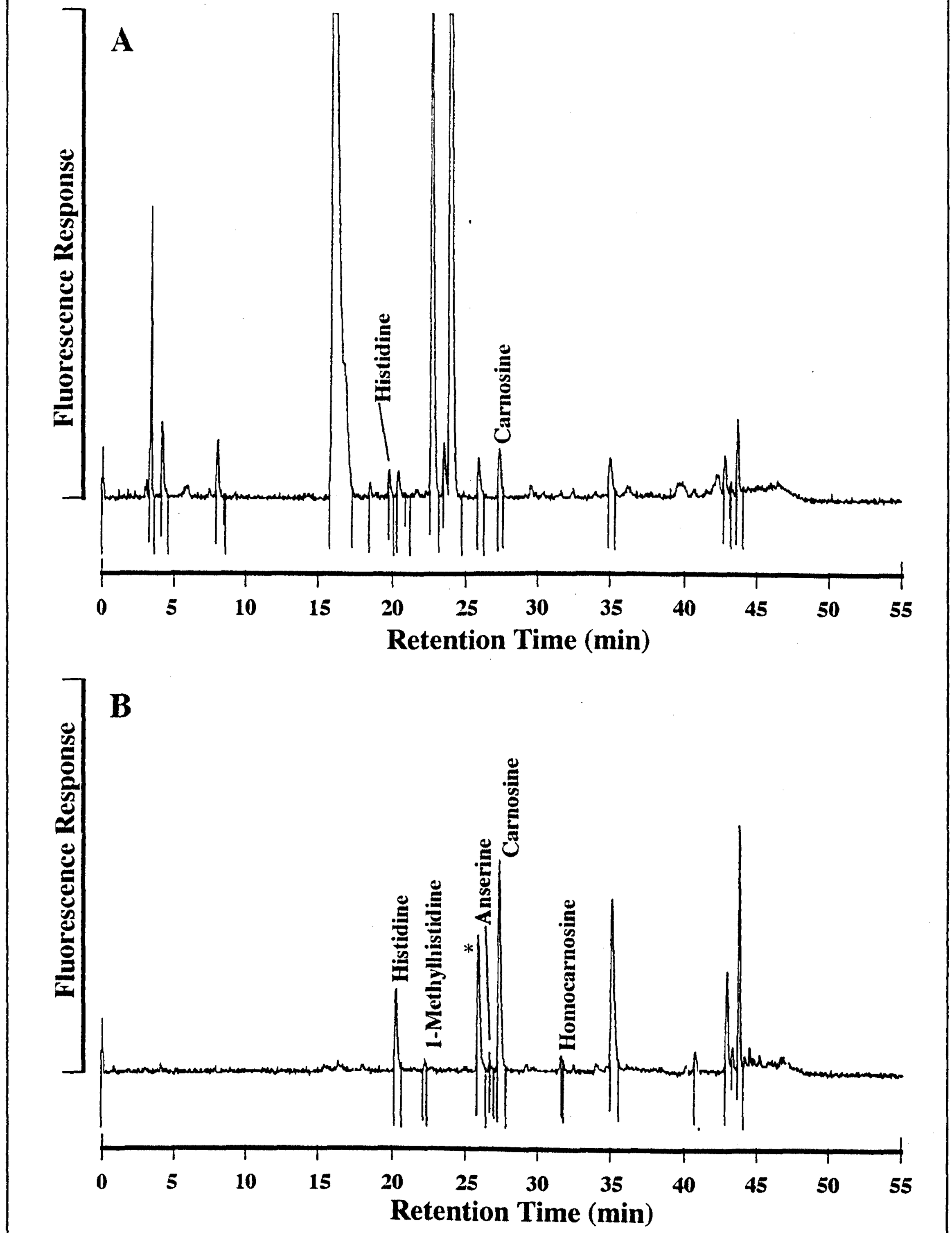

A
Fluorescence Response
Histidine
Carnosine
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)
B
Fluorescence Response
Histidine
1-Methylhistidine
*
Anserine
Carnosine
Homocarnosine
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

Figure 3.6 HPLC separation of a perchloric acid extract of equine middle gluteal muscle post-SPE.

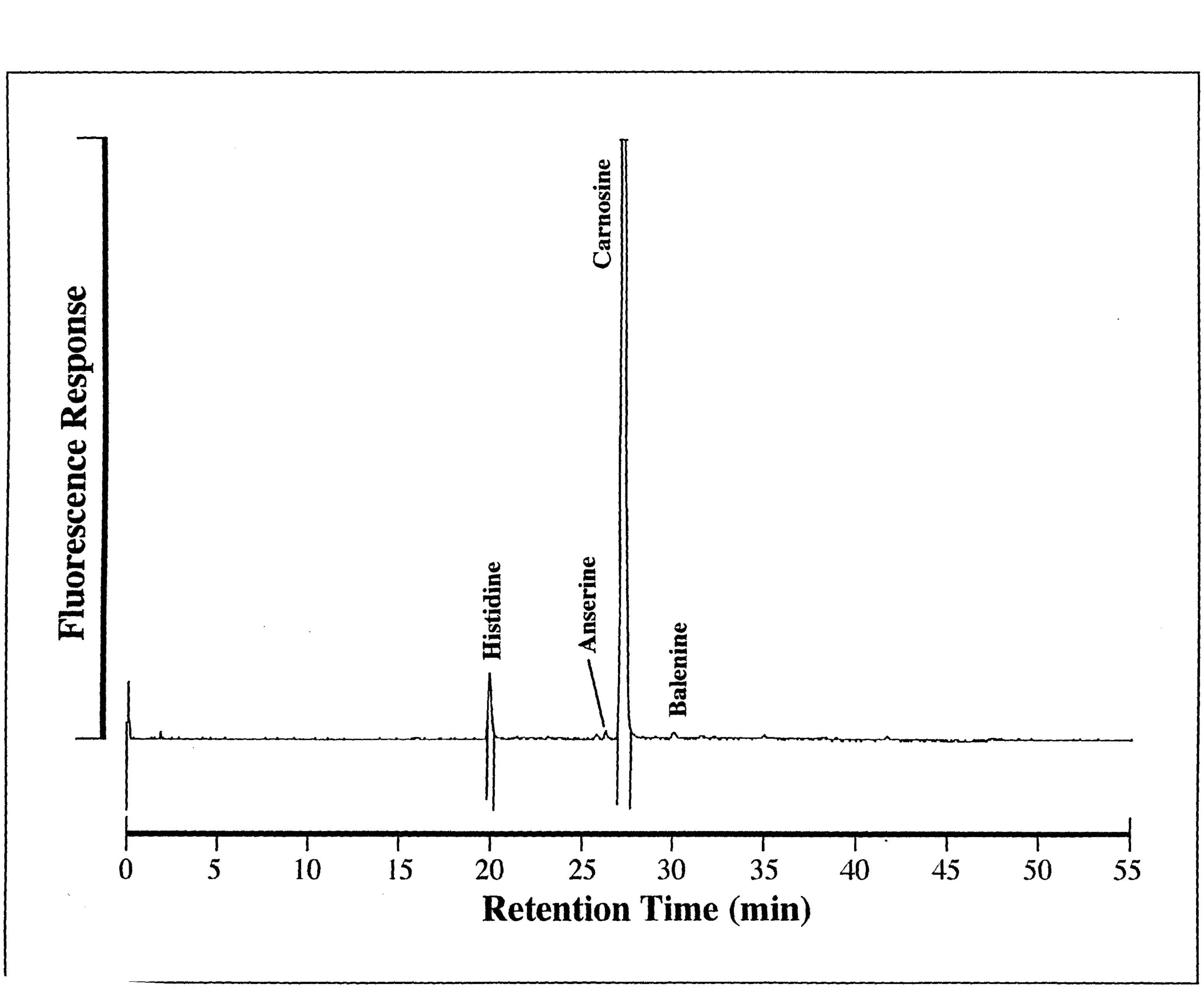

Fluorescence Response
Carnosine
Histidine
Anserine
Balenine
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min)

Table 3.3 Mean recoveries ± CV(%) of the imidazoles from muscle.

Compound	100 mmol kg^{-1} DW	50 mmol kg^{-1} DW	5 mmol kg^{-1} DW	1 mmol kg^{-1} DW
	Recovery ± CV (%)	Recovery ± CV (%)	Recovery ± CV (%)	Recovery ± CV (%)
Histidine	106.0 ± 5.5	101.8 ± 5.0	99.3 ± 3.1	96.0 ± 8.3
1-Methylhistidine	105.1 ± 5.0	109.1 ± 10.8	100.9 ± 11.9	96.9 ± 3.9
3-Methylhistidine	101.7 ± 4.1	103.6 ± 11.1	101.0 ± 11.9	100.8 ± 9.5
Anserine	99.5 ± 10.2	111.1 ± 6.8	99.2 ± 3.2	101.4 ± 6.7
Carnosine	91.1 ± 10.2	109.5 ± 9.2	98.4 ± 11.5	100.8 ± 9.2
Balenine	99.1 ± 8.8	105.1 ± 7.0	99.3 ± 3.3	103.1 ± 6.6
Homocarnosine	100.8 ± 8.1	112.7 ± 9.5	102.5 ± 7.4	115.6 ± 9.6

Table 3.4 Intra-assay precision and accuracy for the determination of imidazoles in muscle.

Compound		Session 1	Session 2	Session 3	Mean
Histidine	Concentration	99.75 ± 0.95	97.88 ± 2.80	96.63 ± 1.19	98.09 ± 1.84 †
	CV	1.0	2.9	1.2	1.7
1-Methylhistidine	Concentration	90.79 ± 6.03	100.29 ± 3.78	103.96 ± 8.88	98.35 ± 6.57 †
	CV	6.7	3.8	8.5	6.3
3-Methylhistidine	Concentration	104.20 ± 2.52	99.84 ± 1.67	98.26 ± 1.55	100.77 ± 1.85 †
	CV	2.4	1.7	1.6	1.9
Anserine	Concentration	103.31 ± 2.01	99.90 ± 1.96	100.65 ± 2.61	101.29 ± 2.21 †
	CV	1.9	2.0	2.6	2.2
Carnosine	Concentration	103.41 ± 1.43	102.72 ± 0.84	102.61 ± 1.62	102.91 ± 1.34 †
	CV	1.4	0.8	1.6	1.3
Balenine	Concentration	100.33 ± 1.47	97.36 ± 0.92	96.55 ± 1.26	98.08 ± 1.24 †
	CV	1.5	0.9	1.3	1.2
Homocarnosine	Concentration	98.08 ± 1.88	96.75 ± 0.97	97.57 ± 2.53	97.47 ± 1.90 †
	CV	1.9	1.0	2.6	1.8

† = Pooled intra-assay SD.

Table 3.5 Inter-assay precision and accuracy for the determination of imidazoles in muscle.

Compound		Day 1	Day 5	Day 10	Mean
Histidine	Concentration	97.88 ± 2.80	101.04 ± 2.07	101.17 ± 2.36	100.03 ± 2.43†
	CV	2.9	2.1	2.3	2.4
1-Methylhistidine	Concentration	100.29 ± 3.78	105.44 ± 2.93	98.35 ± 2.00	101.36 ± 2.99†
	CV	3.8	2.8	2.0	2.9
3-Methylhistidine	Concentration	99.84 ± 1.67	101.54 ± 1.90	100.58 ± 2.43	100.65 ± 2.03†
	CV	1.7	1.9	2.4	2.0
Anserine	Concentration	99.90 ± 1.96	98.98 ± 1.39	100.72 ± 2.14	99.87 ± 1.86†
	CV	2.0	1.4	2.1	1.9
Carnosine	Concentration	102.72 ± 0.84	95.58 ± 2.14	103.36 ± 1.91	100.55 ± 1.73†
	CV	0.8	2.2	1.9	1.7
Balenine	Concentration	97.36 ± 0.92	97.00 ± 2.32	102.44 ± 2.71	98.93 ± 2.13†
	CV	0.9	2.4	2.7	2.2
Homocarnosine	Concentration	96.75 ± 0.97	90.16 ± 3.75	108.36 ± 2.62	98.42 ± 2.70†
	CV	1.0	4.2	2.4	2.7

† = Pooled inter-assay SD.

3.4 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF N-α-ACETYLCARNOSINE IN EQUINE PLASMA

3.4.1. Introduction

Owing to the absence of a free primary amine group in N-α-acetylcarnosine plasma concentrations could not be determined by high-performance liquid chromatography (HPLC) with fluorescence detection following derivatization with *o* -phthaldialdehyde/3-mercaptopropionic acid or fluorescamine. Concentrations of N-α-acetylcarnosine and acetylamino acids have been determined in skeletal muscle, myocardium and brain of several species, including the rat, frog and rabbit. The analytical techniques employed for the analysis included thin layer chromatography (TLC) (Sobue *et al.* 1975), HPLC (O'Dowd *et al.* 1992; O'Dowd *et al.* 1990; O'Dowd *et al.* 1988) and more recently liquid chromatography-mass spectrometry (LC-MS) (Sugahara *et al.* 1994). However, these methods were not developed for the analysis of plasma and with the exception of the latter technique the earlier methods lacked the necessary sensitivity to quantify low concentrations of the compounds of interest in plasma. A new analytical method which combined isocratic reversed-phase HPLC with SPE to provide rapid, selective and sensitive detection of N-α-acetylcarnosine in equine plasma was developed.

3.4.2. Experimental

Instrumentation and reagents

The HPLC system comprised a Hewlett-Packard HP1050 quaternary-gradient solvent delivery system, a Rheodyne 7125 injector (with 200 μl volume sample loop), a HP1040 diode array detector, a Hewlett-Packard Chemstation and Thinkjet printer. A Sepralyte ODS 40 μm (30 x 4.6 mm) sacrificial guard column (Analytichem International, Habor City, CA, USA) and a Rheodyne in-line filter (0.5 μm pore size) were inserted between the pump and the injector.

All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).

Deproteinization and solid-phase extraction

Plasma (250 μl) was deproteinized and extracted with 1.2 *M* perchloric acid in 1.0 *M* phosphoric acid (350 μl), vortex-mixed, centrifuged and the supernatant collected. The precipitate was re-extracted with 1.0 *M* phosphoric acid (400 μl). The supernatants were combined. N-α-acetylcarnosine was isolated from interfering substances by SPE. Plasma extracts (250 μl) were loaded onto 1 ml / 100 mg Isolute PRS (propylsulphonyl) strong cation-exchange columns previously conditioned with methanol (3 x 1000 μl.) and 1.0 *M* phosphoric acid (3 x 1000 μl.). Columns were washed with 50 m*M* potassium dihydrogen phosphate (KH_2PO_4), pH 2.5 (2 x 250 μl) and air-dried for 2 min. The analyte was eluted with 100 m*M* KH_2PO_4, pH 2.5 (2 x 250 μl). A flow-rate of 0.5 ml min^{-1} was used at all times.

Chromatography

HPLC of N-α-acetylcarnosine was performed on a Hypersil ODS (3 μm, 150 x 4.6 mm) analytical column protected by a Hypersil ODS (5 μm, 20 x 4.6 mm) guard column. N-α-acetylcarnosine was eluted using a mobile phase comprising 5 m*M* phosphoric acid and 1 m*M* triethylamine, pH 2.5 in HPLC grade water. The mobile phase was filtered to 0.45 μm and degassed by helium sparging prior to and continually during use. The mobile phase was freshly prepared each day. The flow-rate was 0.8 ml min^{-1} and the injection volume was 200 μl. Detection was by UV absorbance at 220 nm.

Standards

A 1 m*M* stock standard of N-α-acetylcarnosine was prepared by dissolving the required weight in HPLC grade water (10 ml). Working standards over the concentration range 0.1 - 100.0 μM were prepared daily by dilution of the stock standard with 100 m*M* KH_2P0_4 buffer, pH 2.5.

Recoveries study

Plasma was spiked at 800 μM and 0.8 μM with a standard containing N-α-acetylcarnosine, to produce nominal concentrations in the final eluate of 100 and 0.1 μM , assuming recoveries of 100%. Extraction recoveries (n = 5), at each concentration, for the combined deproteinization

and SPE were determined by comparing concentrations found in the final eluates with standards at the nominal concentrations.

Reproducibility study

The reproducibility of the method was assessed by calculation of the intra- and inter-assay accuracy and precision. Plasma was spiked with N-α-acetylcarnosine at 800 μM and 0.8 μM concentrations. The precision of the method was derived from intra- and inter-assay coefficients of variation (CV) of replicate analyses (n = 5) at each concentration. The accuracy of the assay was expressed as: (measured concentration / nominal concentration)·100. Inter-assay CV were determined by analysis on days 1, 5 and 10 over a 10-day period.

Lower detection limit

The lower limit of detection for N-α-acetylcarnosine was quantified by injecting 200 μl of a 10 μM standard which resulted in FSD at a detector sensitivity setting of 10 mAU. The minimum area reliably measurable was 30 area units which approximated to a 5 mm peak at a signal to noise ratio of 3:1. The concentration of the minimum peak was interpolated from the standard curve.

3.4.3. Results and discussion

Chromatography and solid-phase extraction

A method developed for the determination of N-α-acetylcarnosine in tissue samples (O'Dowd *et al.* 1988) which utilized 100 mM sodium dihydrogen phosphate, pH 2.0 buffer as the mobile phase, was applied to the measurement of these analytes in equine plasma. However, this proved unsuccessful. Spiked plasma samples indicated an interfering peak co-eluting with N-α-acetylcarnosine. Furthermore, N-α-acetylcarnosine exhibited poor peak symmetry; the asymmetry factor was 2.56. The degree of interference was reduced and retention time increased by using a mobile phase containing only 5 mM phosphoric acid. The inclusion of triethylamine at 20% of the phosphate concentration further improved peak symmetry; asymmetry factor was 1.07. However, triethylamine concentrations > 1 mM had an adverse effect. The combination

of 5 m*M* phosphoric acid. and 1 m*M* triethylamine resulted in the pH 2.5 solution. No further mobile phase pH adjustment was made and the batch-batch reproducibility of pH was excellent. Analytical columns with several different ODS 3 μm stationary phases were assessed during method development; including Spherisorb, Apex, Primesphere and Hypersil. The latter provided superior performance in terms of resolution and peak symmetry. The retention time for N-α-acetylcarnosine was 5.25 min.

Complete resolution of the N-α-acetylcarnosine peak from the interfering peak was not achieved by column selection alone, and it proved necessary to employ SPE to achieve the sample purity required for accurate quantification of the N-α-acetylcarnosine peak. Despite the absence of a charged terminal amine group on N-α-acetylcarnosine unlike carnosine, the presence of a positive charge on the imidazole ring allowed sufficient retention on cation-exchange sorbents. Optimum retention was obtained on PRS sorbent. The use of 50 and 100 *mM* phosphate buffers for washing the SPE column resulted in a significant reduction in the number of interfering compounds in the final eluate. A comparison of plasma extracts pre- and post SPE is shown in Figure 3.8.

Standards

The standard curve demonstrated a linear relationship between concentration and integrated peak area. The linear regression equations for the N-α-acetylcarnosine standard curve was y = 11.713x - 0.028 (r = 0.999), where y = peak height (mm) and x = concentration (μM).

Lower detection limit

The lower limit of detection in plasma was 0.1 μM.

Recovery and reproducibility studies

Mean (± CV) recoveries of N-α-acetylcarnosine from equine plasma at 800 μM and 0.8 μM concentrations were 93.9 ± 5.0% and 99.7 ± 0.5%, respectively. The intra- and inter-assay

accuracy and precision of the combined SPE and HPLC method at high and low concentrations are given in Table 3.6.

The combined SPE procedure and isocratic reversed-phase HPLC method developed provided a selective, sensitive and reproducible analysis of N-α-acetylcarnosine concentrations in equine plasma.

Figure 3.7. HPLC separation of a of N-α-acetylcarnosine standard.

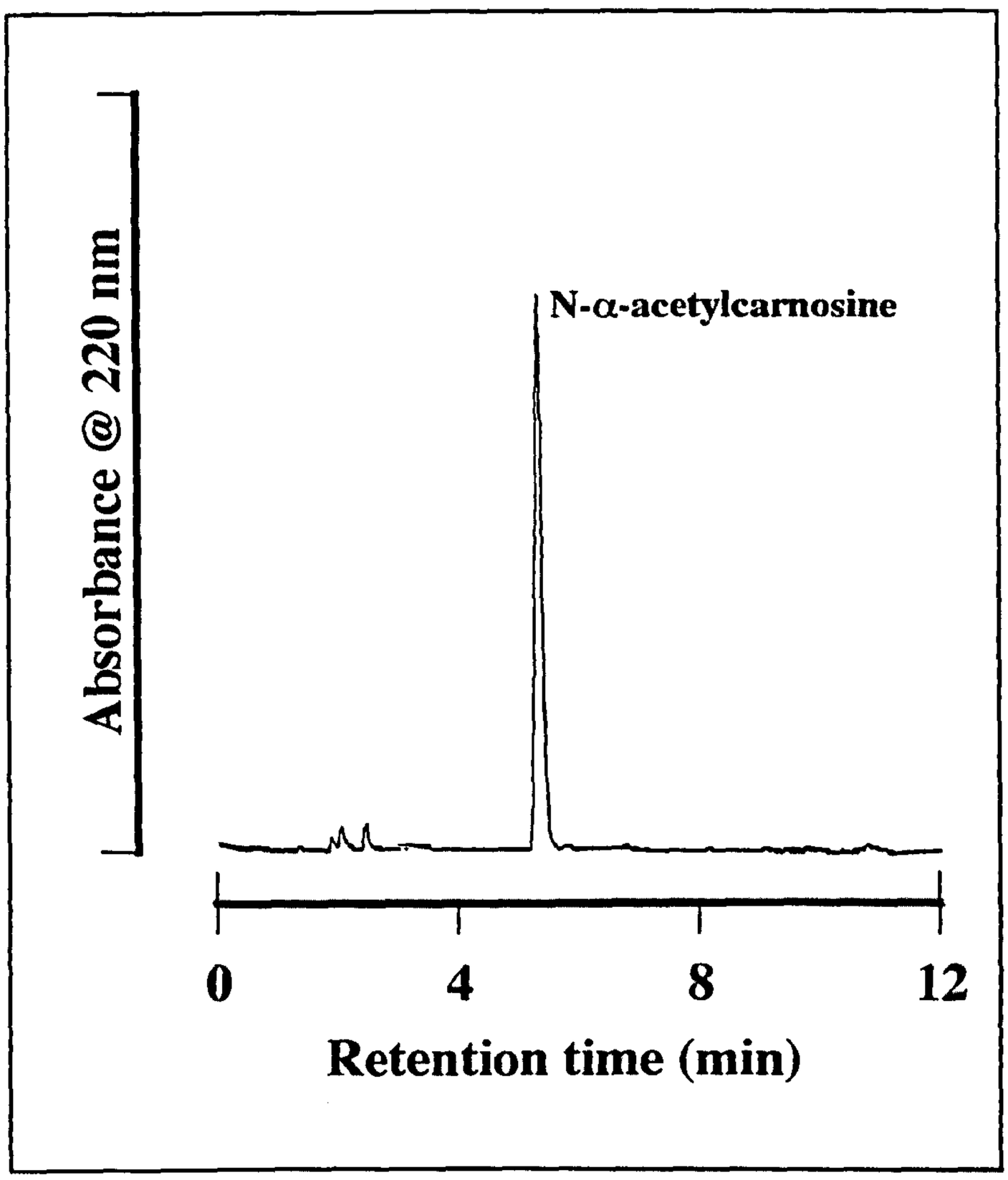

Absorbance @ 220 nm
N-α-acetylcarnosine
0
4
8
12
Retention time (min)

Figure 3.8. HPLC separation of a perchloric acid extract of plasma; A) Pre-SPE. B) Post-SPE.

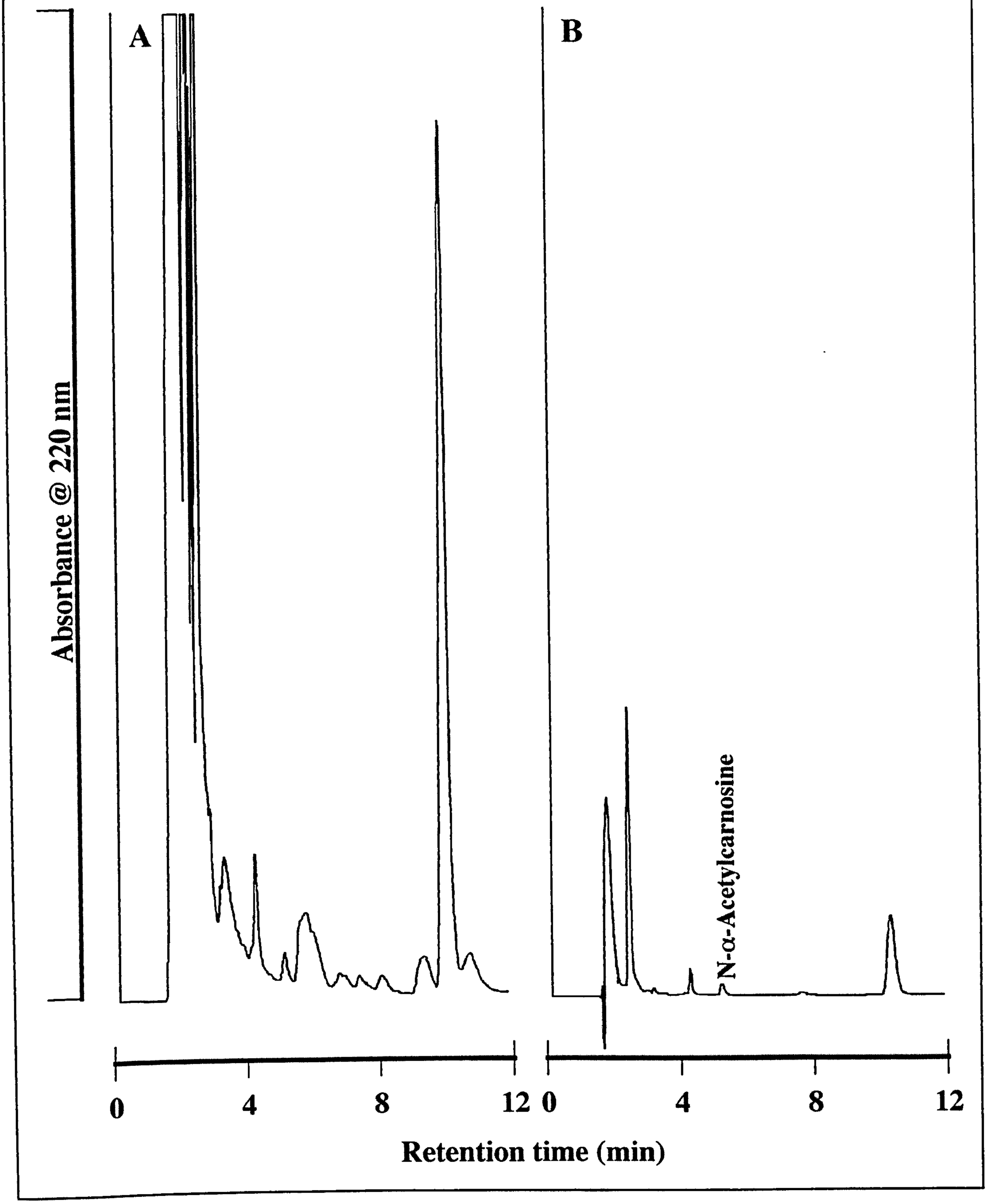

A
B
Absorbance @ 220 nm
N-α-Acetylcarnosine
0
4
8
12
0
4
8
12
Retention time (min)

Table 3.6 Intra- and inter-assay accuracy and precision of the N-α-acetylcarnosine analysis.

Concentration	Intra-assay (n = 5)		Inter-assay (n = 15)	
	Mean ± SD	CV	Mean ± SD	CV
100 μM	98.3 ± 3.3†	3.4	93.9 ± 3.4†	3.6
0.1 μM	100.1 ± 0.1†	0.1	99.8 ± 0.1†	0.1

† = Pooled SD.

CHAPTER 4.

CARNOSINE DISTRIBUTION IN TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE, AND OTHER TISSUES OF THE THOROUGHBRED HORSE.

4.1 INTRODUCTION

Carnosine and the other imidazole dipeptides, with the exception of homocarnosine, occur at high concentrations, often in excess of 100 mmol kg^{-1} DW, within the skeletal muscles of many mammalian species (Crush 1970; Carnegie *et al.* 1983; Plowman and Close 1988). Carnosine also occurs at low millimolar concentrations in other organs such as, the heart, and liver, and tissues of the CNS and gastro-intestinal tract (GIT). Accordingly additional roles, other than acid-base regulation, have been ascribed to carnosine. For example, millimolar concentrations of both carnosine and homocarnosine have been detected in various regions of the central nervous systems of several species (Margolis 1974; Margolis and Grillo 1984). Carnosine is particularly abundant in the olfactory bulb and epithelium of mice and rats (Wideman *et al.* 1978). It has been fairly well established that both carnosine and homocarnosine function as neurotransmitters/neuromodulators in the CNS, and that this role for carnosine is particularly significant within the olfactory pathway (Margolis 1980; Margolis 1981). Owing to the presence of significant amounts of carnosine in certain tissues it has been proposed that carnosine may represent a reservoir of histamine since its catabolism involves hydrolysis to histidine and subsequent decarboxylation to histamine (Flancbaum *et al.* 1990).

Species which possess high intra-muscular concentrations of these dipeptides are generally characterized by an adaptation to either high speed running, such as the horse, or to prolonged periods of hypoxia, such as the whale, where muscle contraction is reliant upon a rapid anaerobic turnover of ATP. Without an integrated system for the maintenance of acid-base balance, the consequential production and accumulation of H^+ ions within the working muscle cells would result in a rapid reduction in intra-cellular pH with a concomitant impairment of the contractile process. The imidazole dipeptides have pK_a values in the range 6.8 to 7.1 enabling them to function as effective proton buffers over the physiological pH range (Bate-Smith 1938; Davey 1960a; Harris *et al.* 1990; Sewell *et al.* 1990). Maintenance of intra-muscular acid-base status during exercise may be as important to athletic performance as the ability to transport oxygen via the cardio-vascular system.

It has been shown that within a species the highest carnosine concentrations are found in skeletal muscles which have the greatest proportion of type II fibres (Tamaki *et al.* 1976; Castelini and Somero 1981). Sewell *et al.* (1990) used multiple linear regression analysis to estimate the carnosine concentrations in types I, IIA and IIB fibres from mixed fibre samples taken from the middle gluteal muscles of horses of different ages and training status. The estimated carnosine concentrations were 21, 86 and 116 mmol kg^{-1} DW in type I, IIA and IIB fibres, respectively. The same approach was used in a comparative study, where muscle samples were obtained by percutaneous biopsy exclusively from young highly trained horses. Estimated carnosine concentrations in this study were found to be higher in type I and IIB fibres, than in the previous study. The estimated carnosine concentrations in type I, IIA and IIB fibres were 54, 85 and 180 mmol kg^{-1} DW, respectively (Sewell *et al.* 1992).

Taurine, a ß-amino acid (2-aminoethanesulphonic acid), is found in most vertebrate tissues and at millimolar concentrations in excitable tissues such as skeletal muscle, heart and CNS (Jacobson and Smith 1968). Skeletal muscle accounts for an estimated 75 % of the total body taurine pool (Stern and Stim 1959). Significant variation in taurine concentration exists between different muscles within and between species. Human muscle taurine concentrations range 8.0 - 21.6 mmol kg^{-1} DW (Zachmann *et al.* 1966) and in the rat values are higher and the range greater; 28.0 - 83.2 mmol kg^{-1} DW (Airaksinen *et al.* 1990). The soleus muscle of the rat, a predominantly slow-twitch muscle, has a two-fold higher taurine content than the extensor digitorum longus, a predominantly fast-twitch glycolytic muscle (Iwata *et al.* 1986). This suggested that an inverse relationship existed between the carnosine and taurine contents of a given muscle which may have been related to the relative proportions of type I (slow-twitch) and type II (fast-twitch) fibres within a given muscle. Subsequent studies in both young highly trained horses and older horses of variable training status indicated that taurine concentrations in equine skeletal muscle were strongly correlated to type I % FSA ($p < 0.001$, $r = 0.94$) and that type I fibres were estimated to contain 35.4 - 45.4 mmol kg^{-1} DW of taurine, whereas type IIA fibres contained only 4.5 - 7.9 mmol kg^{-1} DW, and taurine appeared to be absent from type IIB fibres (Dunnett *et al.* 1992).

4.2 STUDY A: CARNOSINE CONCENTRATIONS AND CARNOSINASE ACTIVITIES IN OTHER EQUINE TISSUES

4.2.1 Objectives

The aims of this study were to determine the carnosine concentrations and carnosinase activities in various equine skeletal muscles and other tissues.

4.2.2 Experimental methodology

Sampling procedure

Samples from tissues were collected at post mortem, as described in Chapter 2, from eighteen thoroughbred horses aged from 7 months to 4 years and from two foetuses.

Analytical methods

Tissue extracts were analysed for carnosine and other imidazole concentrations by high-performance liquid chromatography as described in Chapter 3 (Dunnett and Harris 1995). Tissue carnosinase activity was determined by a modification of the method of Lenney (1990), as described in Chapter 2.

4.2.3 Results

Samples were collected from the following tissues; myocardium, kidney, liver, spleen, cerebellum, medulla, lung, stomach, small intestine, colon and rectum. Mean (± SD) tissue concentrations of carnosine and histidine, and tissue carnosinase activities are presented in Table 4.1. Mean values for carnosine concentrations determined in middle gluteal and internal intercostal muscle samples are included for comparative purposes. Carnosine was present at much lower concentrations in myocardium, kidney, liver, spleen, lung and tissues from the CNS and GIT, than those found in typical locomotory skeletal muscles such as the middle gluteal. The carnosine concentration in the diaphragm is two to three-fold lower than the concentration typically found in mixed fibre samples from the middle gluteal muscle. However, the diaphragm carnosine concentration is similar to the value found in type I skeletal muscle fibres. Carnosine

concentrations measured in cardiac muscle (myocardium) and smooth muscle (small intestine, colon, stomach and rectum) were twenty-fold or more lower than those found in diaphragm.

Anserine and balenine were also detected in equine skeletal muscle and diaphragm at concentrations two to three orders of magnitude lower than for carnosine. Anserine was present at 0.66 ± 0.27 and 0.66 ± 0.13 mmol kg^{-1} DW in the middle gluteal and intercostal muscles, respectively, and at 0.82 ± 0.10 mmol kg^{-1} DW in the diaphragm. Balenine was present at 0.20 ± 0.06 and 0.10 ± 0.05 mmol kg^{-1} DW in the middle gluteal and intercostal muscles, respectively, and at 0.08 ± 0.03 mmol kg^{-1} DW in the diaphragm. Anserine and balenine were not detected in other tissues. Homocarnosine was detected only in tissues from the CNS. It was present at concentrations of 0.09 ± 0.09 and 0.04 mmol kg^{-1} DW in cerebellum and medulla, respectively. Trace amounts only of l-methylhistidine were present in skeletal muscle and it was not detected in other tissues. No free 3-methylhistidine was detected in muscle samples although it is a constituent of the contractile proteins, actin and myosin.

Tissue carnosinase activities were highest in the kidney, and other tissues exhibiting relatively high activities were, the small intestine, spleen and lung. Carnosinase activity in the middle gluteal muscle, internal intercostal muscle and the diaphragm was below the lower limit of quantification of the assay (1.4 μmol g^{-1} h^{-1} DW).

Table 4.1 Carnosine and histidine concentrations, and carnosinase activities in various tissues of the thoroughbred horse.

Tissue	n	Carnosine (mmol kg^{-1} DW)	Histidine (mmol kg^{-1} DW)	Carnosinase (μmol g^{-1} h^{-1} DW)
Middle gluteal	7	107.81 ± 12.87	0.25 ± 0.05	ND
Internal intercostal	3	63.70 ± 16.16	0.39 ± 0.01	ND
Diaphragm	5	38.62 ± 5.09	0.60 ± 0.36	ND
Stomach	2	2.43 ± 0.80	1.30 ± 0.06	4.2 ± 3.3
Myocardium	6	2.22 ± 1.21	0.56 ± 0.06	3.0 ± 2.5
Rectum	1	1.27	1.57	4.4
Small intestine	11	0.93 ± 0.47	1.44 ± 0.41	22.9 ± 17.7
Colon	1	0.58	1.46	3.6
Lung	2	0.43 ± 0.05	0.86 ± 0.52	13.7 ± 15.1
Kidney	7	0.13 ± 0.04	0.33 ± 0.10	604.0 ± 24.2
Spleen	3	0.12 ± 0.07	0.61 ± 0.14	23.7 ± 7.3
Liver	8	0.11 ± 0.05	0.90 ± 0.43	5.6 ± 2.0
Cerebellum	9	0.04 ± 0.03	0.25 ± 0.10	1.8 ± 1.4
Medulla	1	0.04	0.40	ND

ND = Not detected

4.3 STUDY B: CARNOSINE AND TAURINE CONTENTS IN TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE OF THE NORMAL THOROUGHBRED HORSE.

Dunnett and Harris (1995a)

4.3.1 Objectives

The aims of the present study was to determine by direct measurement the carnosine and taurine concentrations in type I, type IIA and type IIB fibres from the middle gluteal muscle of the thoroughbred horse, and to assess whether taurine : carnosine ratios could be used as a biochemical means for determining fibre type in individual muscle fibres.

4.3.2 Experimental methodology

Protocol and sampling procedure

Muscle samples from the left or right middle gluteal muscle were collected at post mortem from 5 thoroughbred horses (3 colts, 2 geldings) which had no history of muscle disorders. The horses were aged between 2 and 13 years and had a variety of training backgrounds. However, all the horses had been out of training for at least 6 months. All of the horses were euthanased having failed to respond to treatment for a variety of chronic conditions. One entire middle gluteal muscle was removed from each horse and samples were collected, as described in detail in Chapter 2. Owing to the relatively low abundance of type I fibres within equine middle gluteal muscle, the number of individual type I fibres retrieved was maximized by selecting some of the darker (more red) samples for dissection. Individual muscle fibres were dissected from freeze-dried muscle samples as described in Chapter 2.

Histochemistry

Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously (Chapter 2).

Individual muscle fibre analysis

Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).

Statistics

Data on the mean carnosine and taurine concentrations of each fibre type are given together with a pooled estimate of the within-horse standard deviation (SD_p). SD_p was calculated using the equation

$$SD_p = \sqrt{(\Sigma SSQ_h / \Sigma n_h\text{-}1)}$$

where SSQ_h is the sum of squares calculated within each horse and n_h-1 is the degrees of freedom upon which this value is based. Analysis of carnosine and taurine distribution in type I, IIA and IIB fibres was performed by normalization of individual values which enabled data from all horses to be combined. Individual values were divided by the mean value for that horse and the results were normalized by multiplying by the overall mean value for all horses. Type I, IIA and IIB fibres were treated independently. One-factor ANOVA was used to identify significant differences in mean (± SD) values for carnosine and taurine concentrations, and taurine : carnosine ratios between type I, IIA and IIB fibres. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at $p < 0.05$.

4.3.3 Results

Carnosine and taurine concentrations were determined in a total of 324 individual muscle fibres taken from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) at post mortem. The number of fibres and mean (± SD) carnosine and taurine concentrations in type I, IIA and IIB muscle fibres for individual horses, and mean values (± SD_p) for all horses combined are given in Tables 4.2 and 4.3, respectively. No carnosine was detected in the type I fibres of one horse (EX), however, this may have been due to an unrepresentative population as only 2 type I fibres were found in the samples from this horse. Distribution plots of carnosine and

taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of the individual horses are given in Figures 4.1 and 4.2, respectively. Carnosine concentrations were significantly different between the three fibre types ($p < 0.001$). The overall mean ($\pm$ SD_p) carnosine concentrations in type I, type IIA and type IIB fibres were 24.9 ± 6.4 , 94.8 ± 6.8 and 104.3 ± 11.9 mmol kg^{-1} DW, respectively.

There was a significant difference in taurine concentration between type I, and type IIA and IIB fibres ($p < 0.001$), but not between type IIA and type IIB fibres ($p > 0.05$). The overall mean ($\pm$ SD_p) taurine concentrations in type I, type IIA and type IIB fibres were 54.3 ± 8.3 , 2.8 ± 2.1 and 1.8 ± 1.9 mmol kg^{-1} DW, respectively. Taurine: carnosine ratios for individual horses and overall mean ($\pm$ SD_p) values are given in Table 4.4. Taurine: carnosine ratios between type I fibres, and type IIA and IIB fibres were significantly different ($p < 0.001$). There was however, no significant difference between type IIA and type IIB fibres ($p > 0.05$). It was therefore not possible to discriminate between type IIA and IIB fibres by use of the taurine : carnosine ratio. Frequency distribution plots of normalized carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.3 and 4.4, respectively.

Table 4.2 Individual mean (± SD) carnosine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SD_p) concentrations for all horses.

Horse	Carnosine, mmol kg^{-1} DW (number of fibres)					
	Type I		Type IIA		Type IIB	
FO	25.5 ± 6.5	(22)	85.7 ± 24.8	(14)	93.1 ± 31.6	(16)
TI	12.2 ± 6.7	(53)	99.9 ± 10.4	(7)	110.7 ± 14.2	(16)
DP	27.3 ± 5.9	(50)	89.7 ± 10.3	(6)	96.6 ± 12.9	(30)
GI	22.2 ± 4.0	(27)	87.6 ± 5.8	(14)	102.8 ± 14.6	(13)
EX	0.0 ± 0.0	(2)	111.0 ± 17.9	(18)	118.5 ± 24.0	(36)
Mean ± SD_p	20.6 ± 6.0	(154)	94.8 ± 16.7	(59)	104.3 ± 20.7	(111)

SD_p Pooled within-horse standard deviation

Table 4.3 Individual mean (± SD) taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SD_p) concentrations for all horses.

Horse	Taurine, mmol kg^{-1} DW (number of fibres)					
	Type I		Type IIA		Type IIB	
FO	62.6 ± 9.0	(22)	3.5 ± 7.0	(14)	2.8 ± 5.7	(16)
TI	54.9 ± 15.7	(53)	1.7 ± 2.9	(7)	0.5 ± 1.8	(16)
DP	62.7 ± 11.8	(50)	2.7 ± 4.6	(6)	3.0 ± 3.3	(30)
GI	69.8 ± 8.3	(27)	2.0 ± 1.6	(14)	0.2 ± 0.7	(13)
EX	58.4 ± 3.5	(2)	3.9 ± 6.0	(18)	2.8 ± 3.1	(36)
Mean ± SD_p	61.2 ± 12.5	(154)	2.8 ± 5.2	(59)	1.8 ± 3.4	(111)

SD_p Pooled within-horse standard deviation.

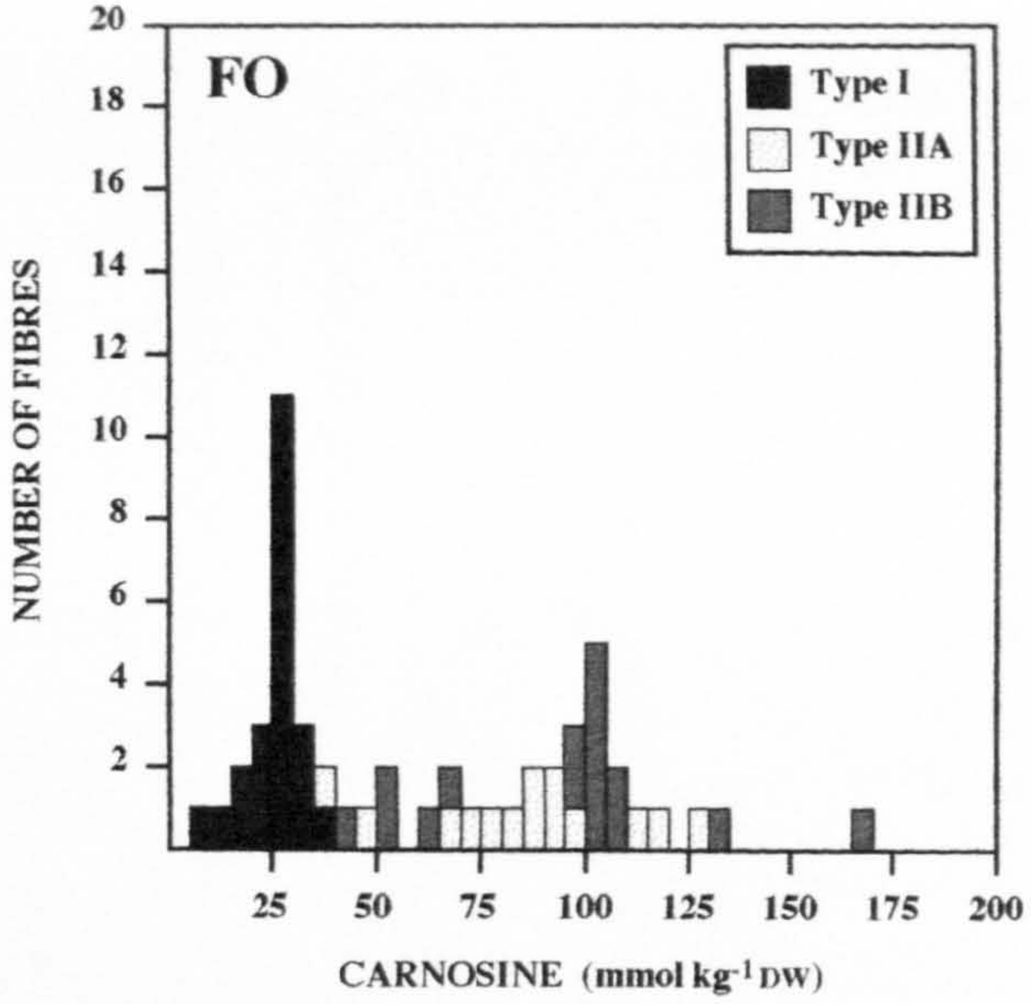
Table 4.4 Individual mean (± SD) taurine : carnosine ratios in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SD_p) concentrations for all horses.

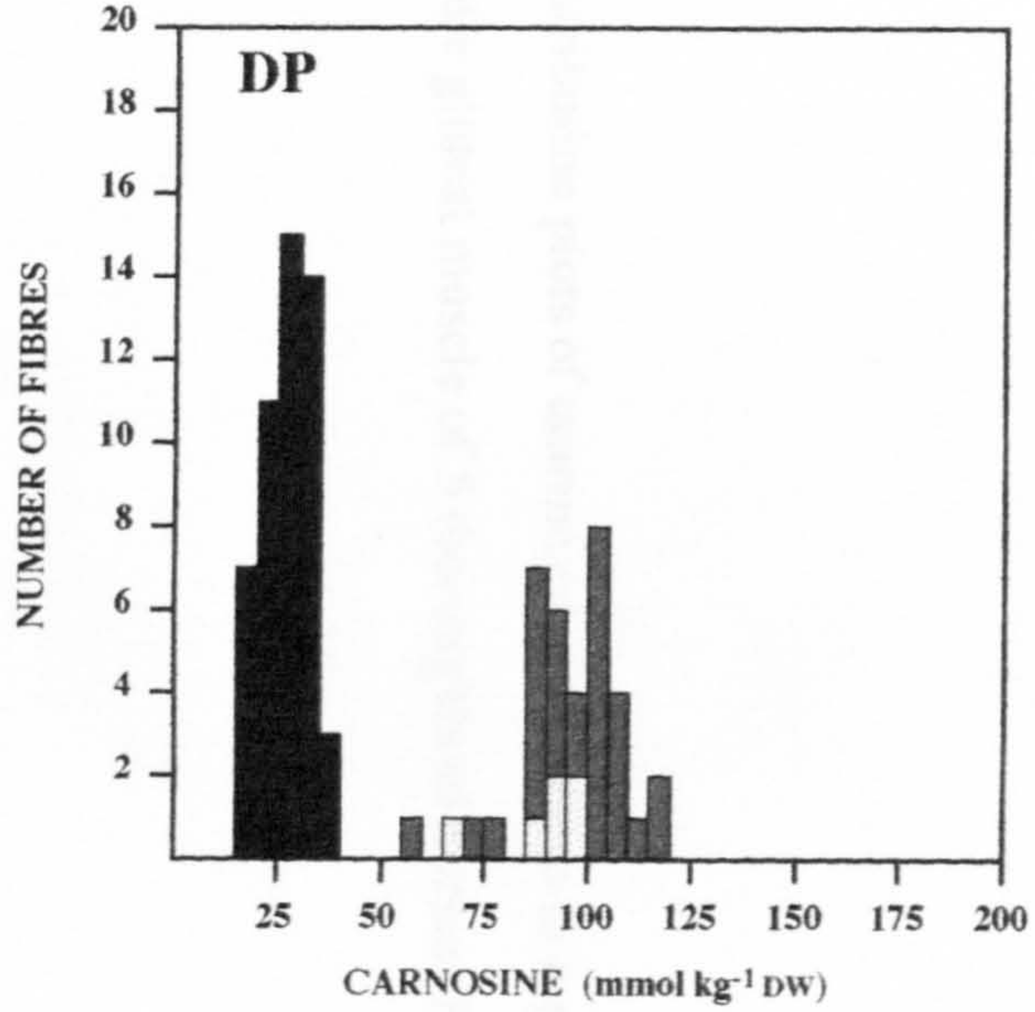
Horse	Taurine : Carnosine ratio		
	Type I	Type IIA	Type IIB
FO	2.573 ± 0.564	0.057 ± 0.121	0.045 ± 0.093
TI	5.559 ± 2.872	0.017 ± 0.031	0.005 ± 0.019
DP	2.331 ± 0.331	0.030 ± 0.050	0.034 ± 0.043
GI	3.202 ± 0.494	0.022 ± 0.019	0.002 ± 0.007
EX	LARGE†	0.037 ± 0.057	0.024 ± 0.027
Mean ± SD_p	3.646 ± 2.241†	0.033 ± 0.029	0.022 ± 0.026

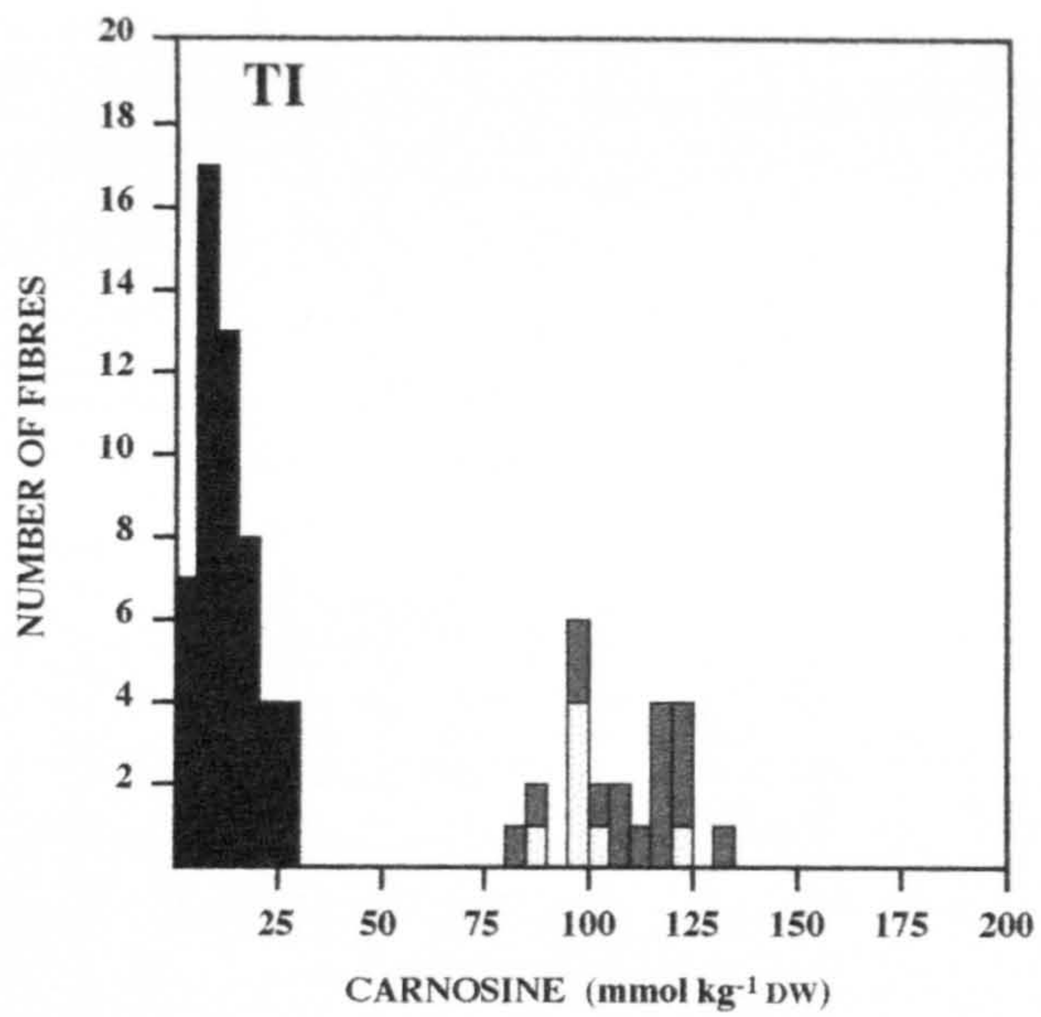
SD_p Pooled sithin-horse standard deviation

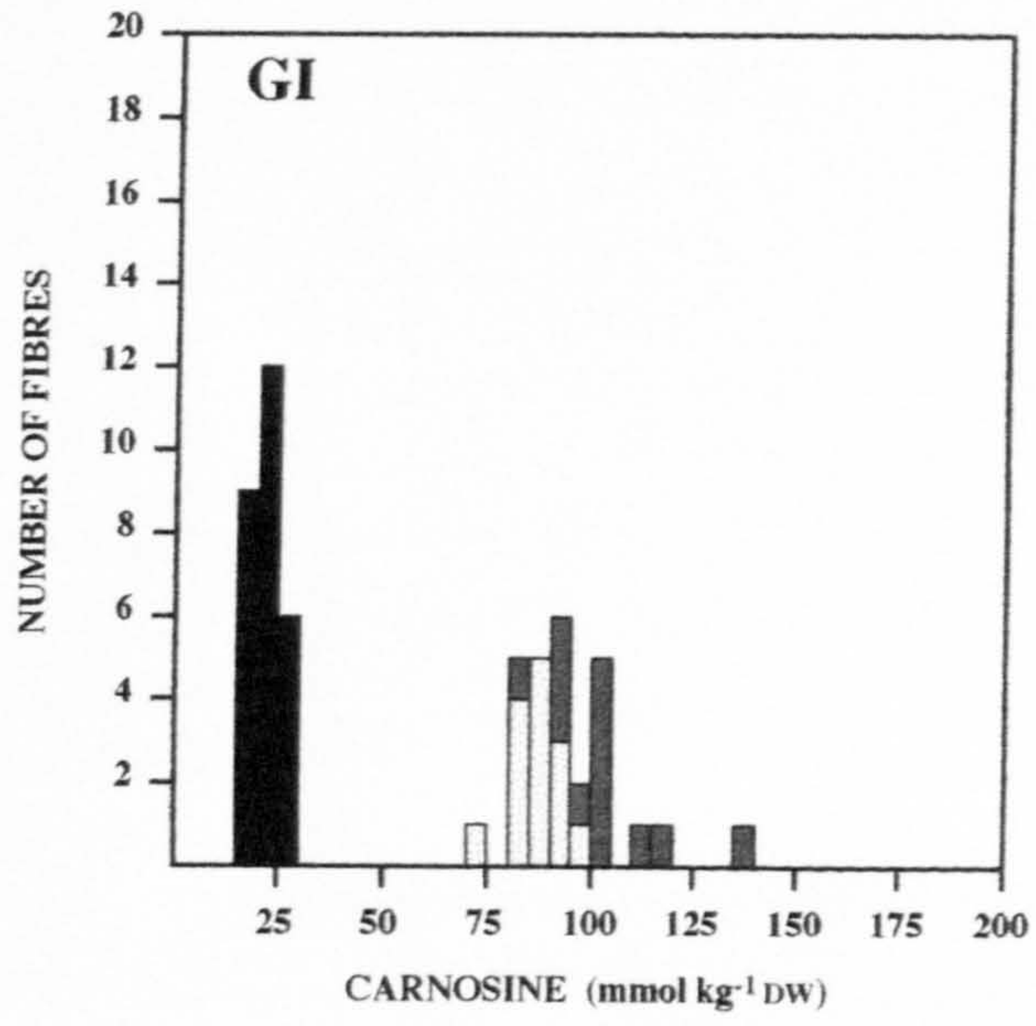
† Carnosine was not detected in EX type I fibres. Mean calculated from 4 horses (EX excluded).

Figure 4.1 Individual distribution plots of carnosine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX).


FO
Type I
Type IIA
Type IIB
NUMBER OF FIBRES
CARNOSINE (mmol kg-1 DW)


DP
NUMBER OF FIBRES
CARNOSINE (mmol kg-1 DW)


TI
NUMBER OF FIBRES
CARNOSINE (mmol kg-1 DW)


GI
NUMBER OF FIBRES
CARNOSINE (mmol kg-1 DW)

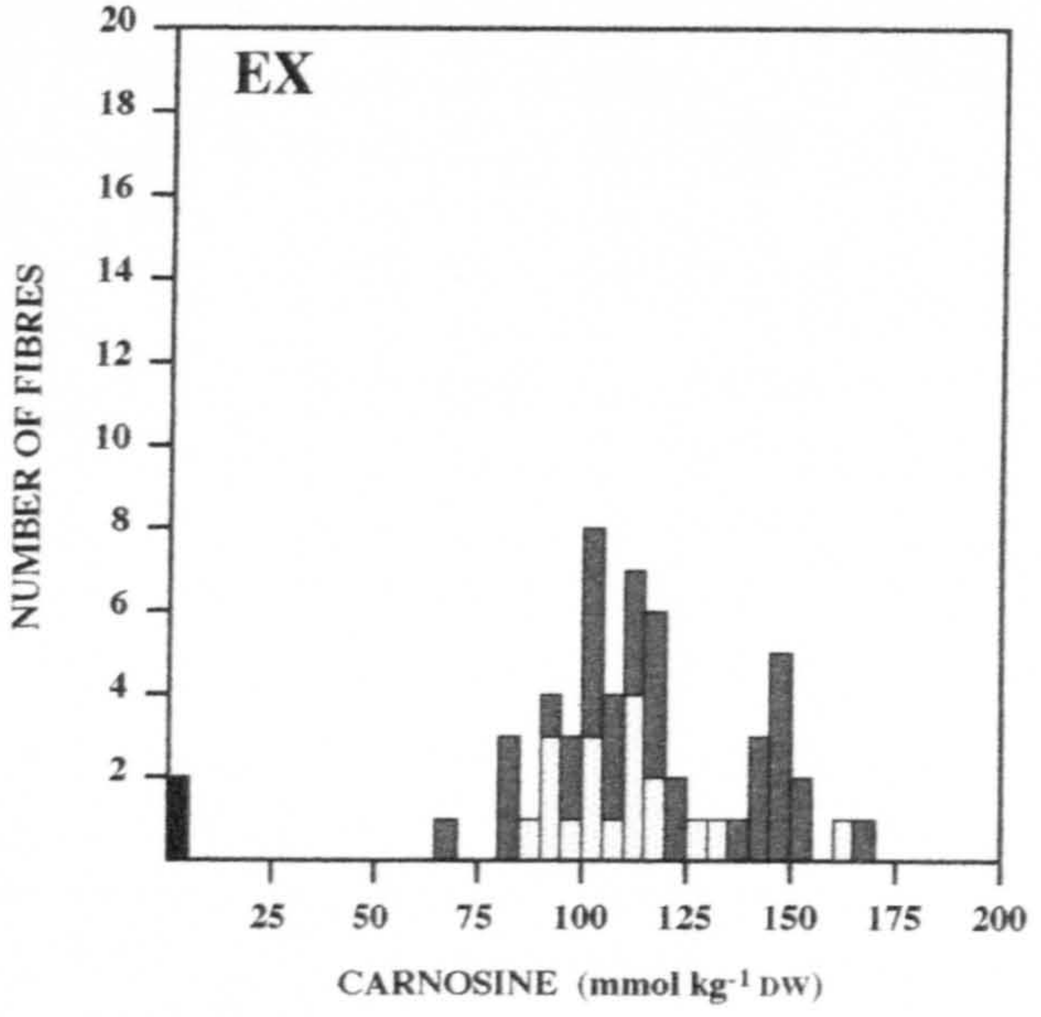
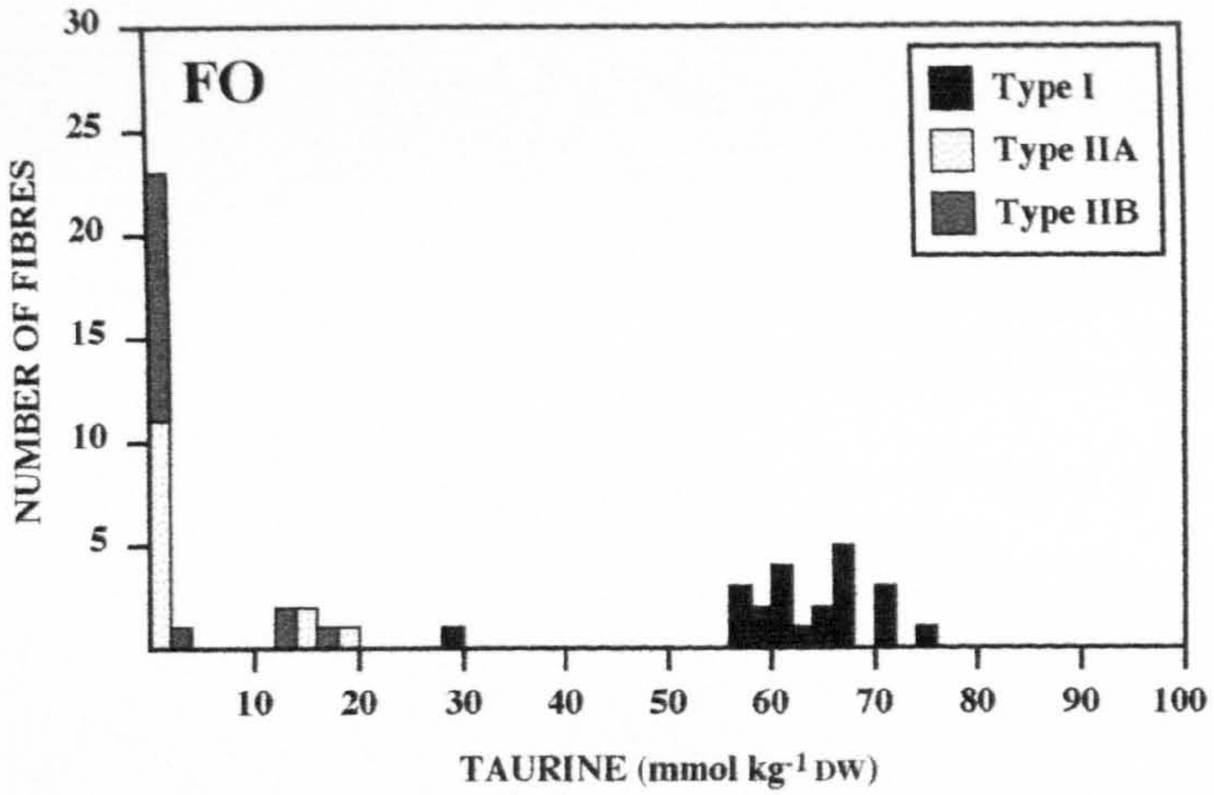
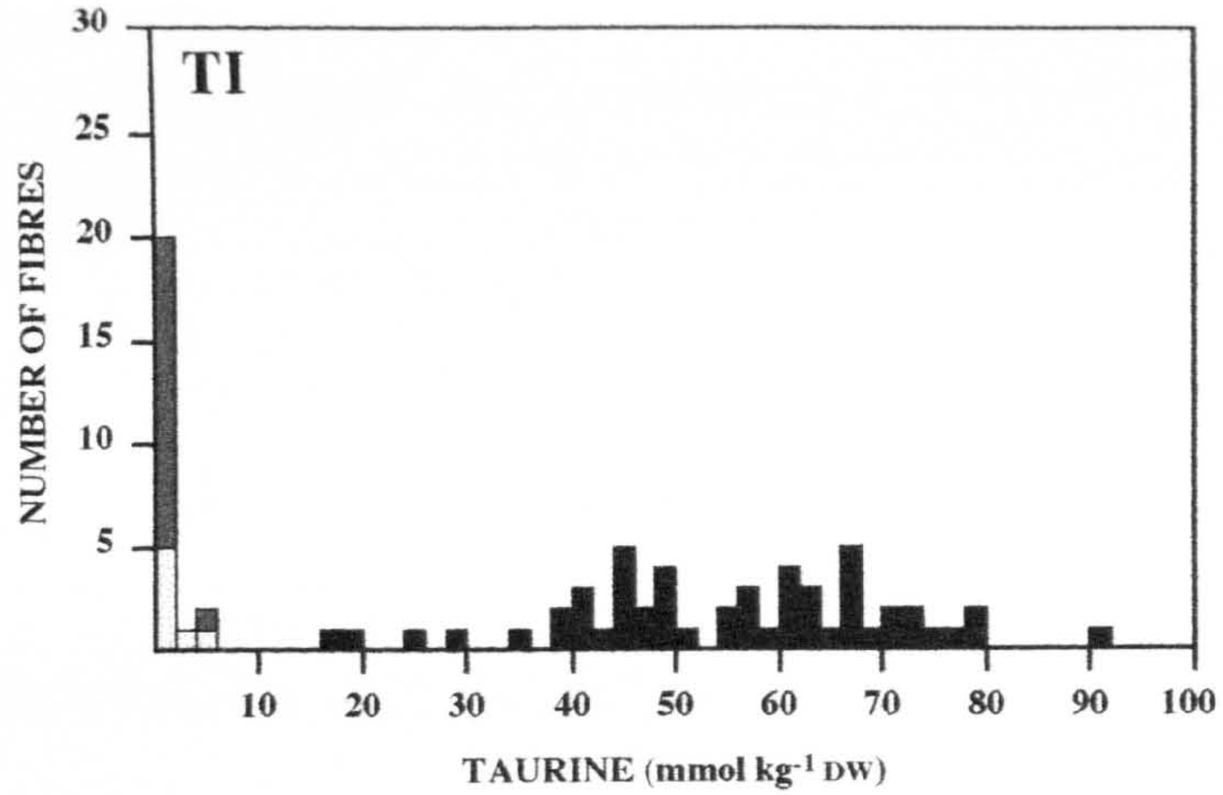
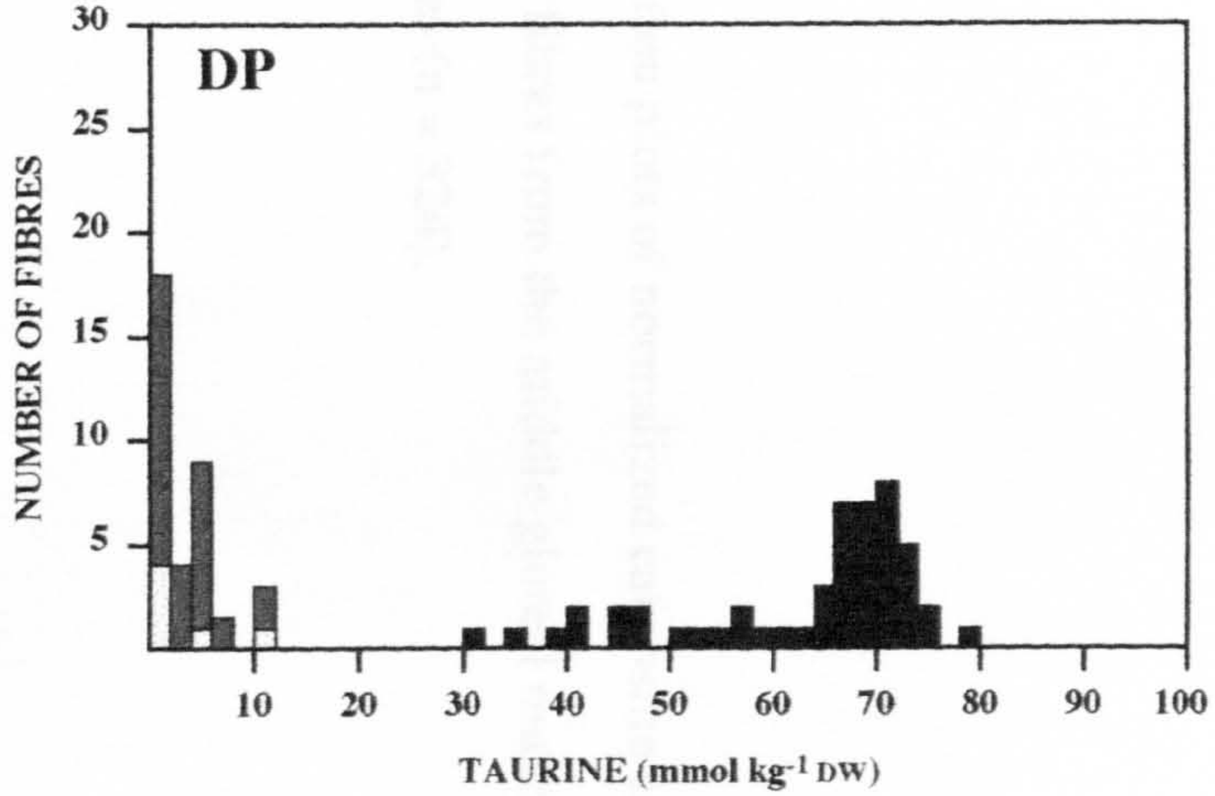
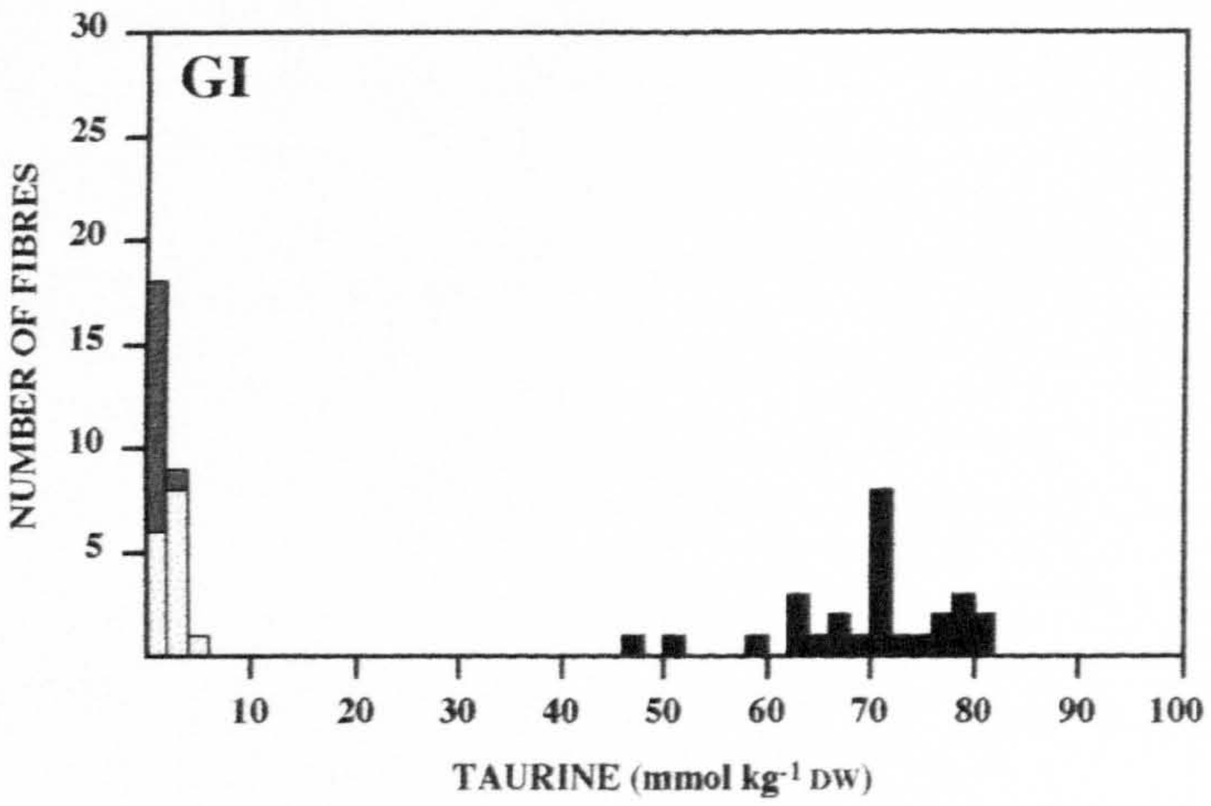

EX
NUMBER OF FIBRES
CARNOSINE (mmol kg-1 DW)

Figure 4.2 Individual distribution plots of taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX).


FO
Type I
Type IIA
Type IIB
NUMBER OF FIBRES
TAURINE (mmol kg-1 DW)


TI
NUMBER OF FIBRES
TAURINE (mmol kg-1 DW)


DP
NUMBER OF FIBRES
TAURINE (mmol kg-1 DW)


GI
NUMBER OF FIBRES
TAURINE (mmol kg-1 DW)

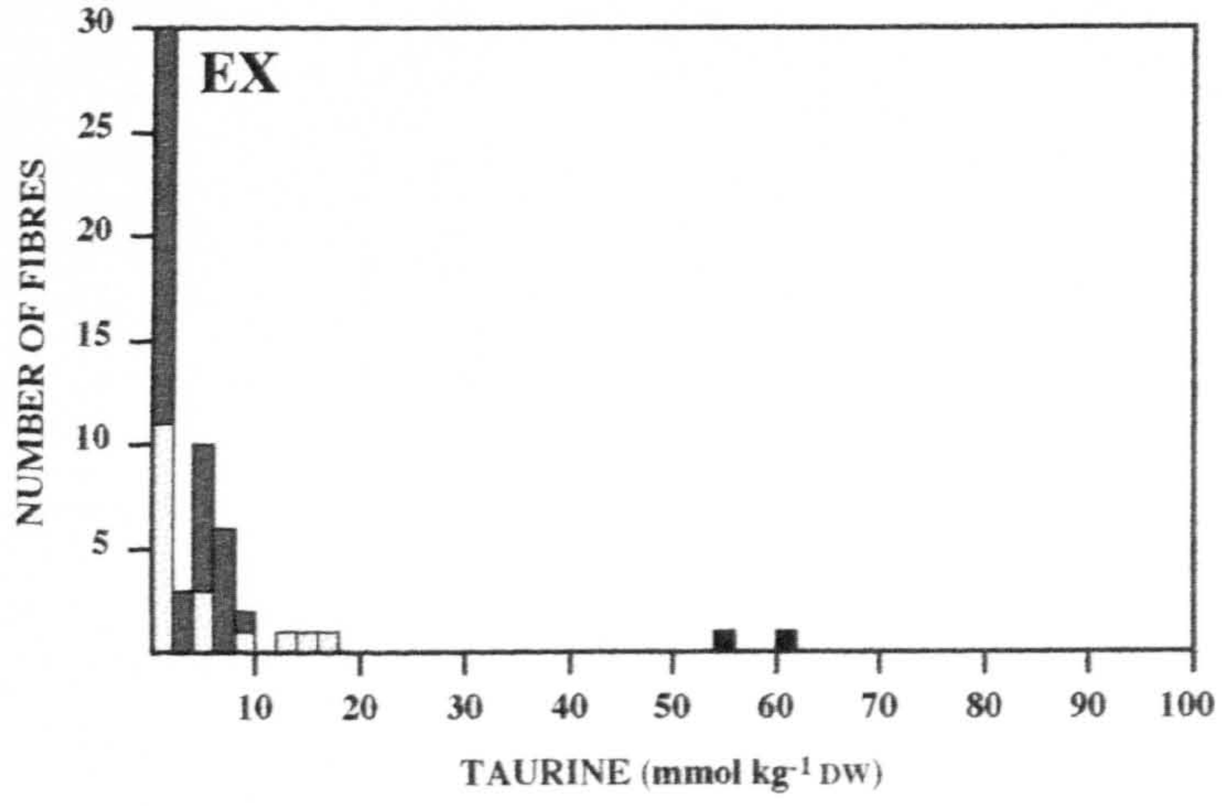

EX
NUMBER OF FIBRES
TAURINE (mmol kg-1 DW)

Figure 4.3 Frequency distribution plots of normalized carnosine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 5 normal thoroughbred horses (n = 324).

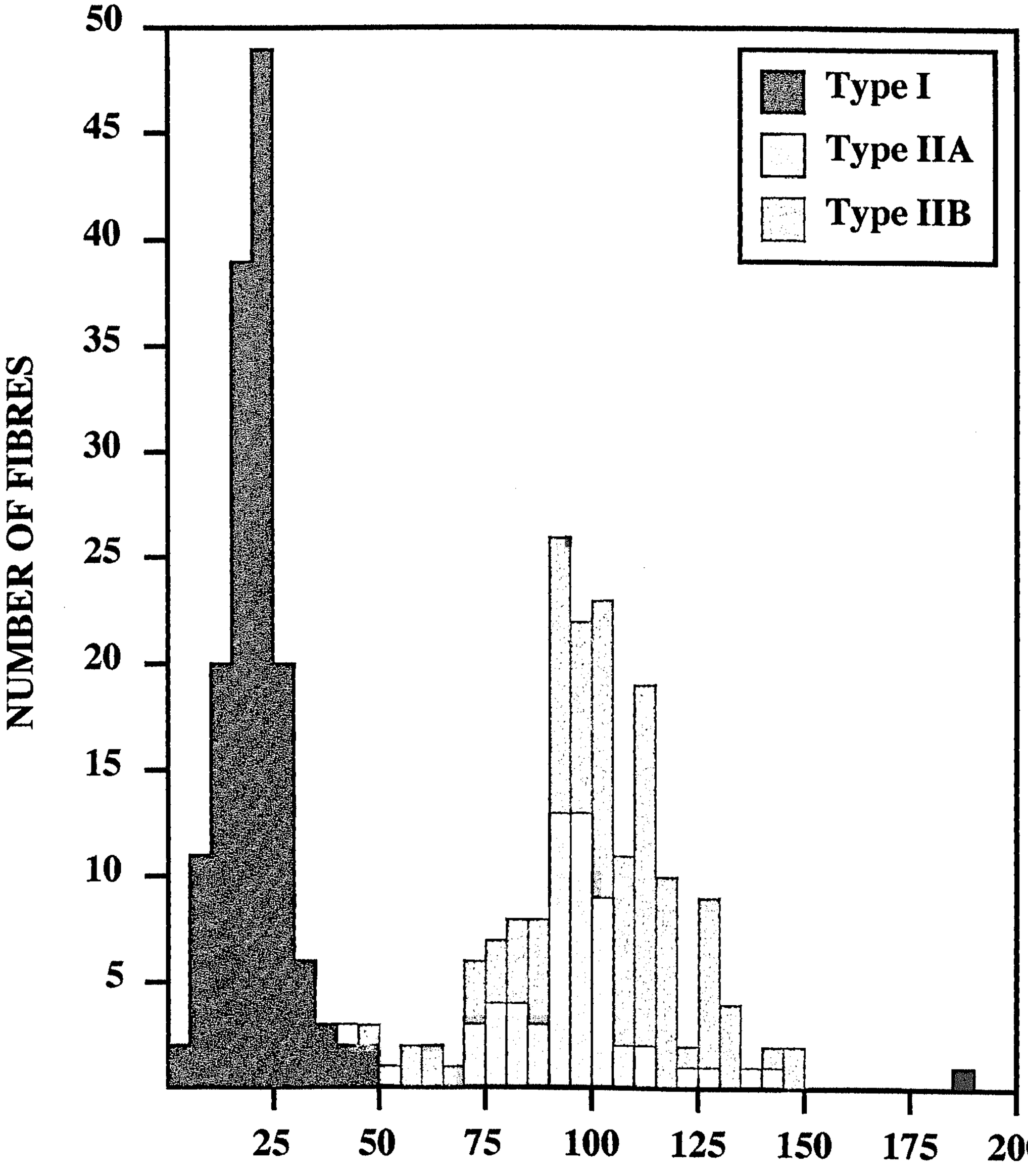
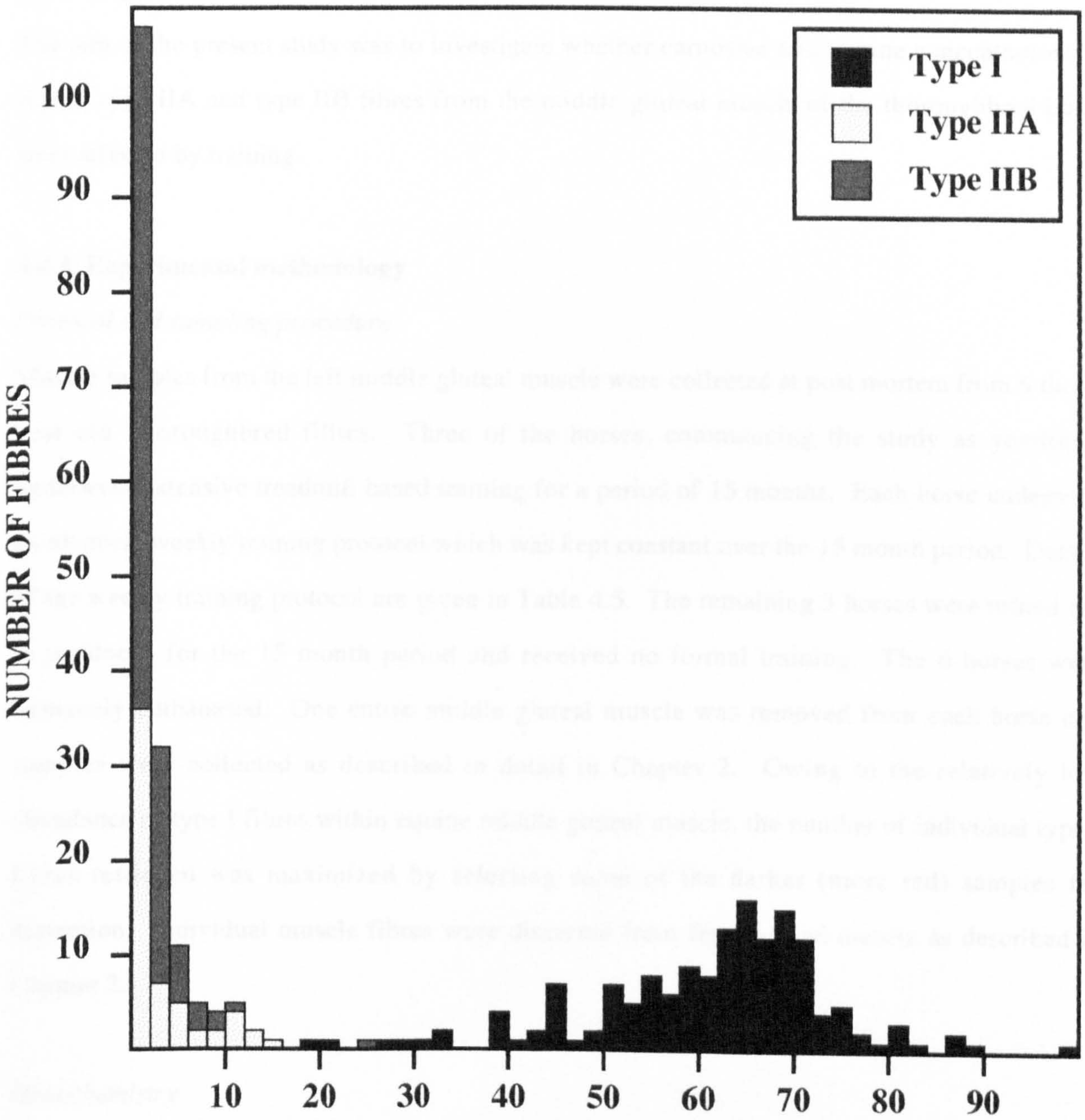

NUMBER OF FIBRES
50
45
40
35
30
25
20
15
10
5
Type I
Type IIA
Type IIB
25
50
75
100
125
150
175
200
CARNOSINE (mmol kg-1 DW)

Figure 4.4 Frequency distribution plots of normalized taurine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 5 normal thoroughbred horses (n = 324).


Type I
Type IIA
Type IIB
NUMBER OF FIBRES
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
TAURINE (mmol kg-1 DW)

4.4 STUDY C: CARNOSINE AND TAURINE CONTENTS OF TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE OF THE UNTRAINED AND TRAINED THOROUGHBRED HORSE.

4.4.1 Objectives

The aim of the present study was to investigate whether carnosine and taurine concentrations in type I, type IIA and type IIB fibres from the middle gluteal muscle of the thoroughbred horse were affected by training.

4.4.2 Experimental methodology

Protocol and sampling procedure

Muscle samples from the left middle gluteal muscle were collected at post mortem from 6 three-year-old Thoroughbred fillies. Three of the horses, commencing the study as yearlings, underwent extensive treadmill based training for a period of 15 months. Each horse underwent an identical weekly training protocol which was kept constant over the 15 month period. Details of the weekly training protocol are given in Table 4.5. The remaining 3 horses were turned-out in paddocks for the 15 month period and received no formal training. The 6 horses were humanely euthanased. One entire middle gluteal muscle was removed from each horse and samples were collected as described in detail in Chapter 2. Owing to the relatively low abundance of type I fibres within equine middle gluteal muscle, the number of individual type I fibres retrieved was maximized by selecting some of the darker (more red) samples for dissection. Individual muscle fibres were dissected from freeze-dried muscle as described in Chapter 2.

Histochemistry

Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously (Chapter 2).

Table 4.5 Weekly training protocol

Day	Walk	Trot	Canter / Gallop (treadmill)
1	40 min	-	5 min @ 12 m s^{-1} (3% incline)
2	40 min	10 min	-
3	40 min	-	2 x 90 s @ 13 & 14 m s^{-1} (3% incline)
4	40 min	10 min	-
5	40 min	-	3 x 70 s @ 12, 13 & 14 m s^{-1} (3% incline)
6	40 min	10 min	-
7	40 min	-	-

Individual muscle fibre analysis

Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).

Statistics

Analysis of carnosine and taurine distribution in type I, IIA and IIB fibres was performed by normalization of individual values which enabled data from all horses to be combined. Individual values were divided by the mean value for that horse and the results were normalized by multiplying by the overall mean value for all untrained or trained horses. Type I, IIA and IIB fibres were treated independently. One-factor ANOVA was used to identify significant differences in mean ($\pm$ SD_p) values for carnosine and taurine concentrations in type I, IIA and IIB fibres between untrained and trained horses. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at $p < 0.05$.

4.4.3 Results

Carnosine and taurine concentrations were determined in a total of 468 individual muscle fibres (untrained, n = 239; trained, n = 229) taken from the middle gluteal muscle of 6 thoroughbred horses at post mortem. The number of fibres and mean ($\pm$ SD) carnosine and taurine concentrations in type I, IIA and IIB muscle fibres for individual horses, and overall mean values ($\pm$ SD_p) for untrained and trained horses are given in Tables 4.6 and 4.7, respectively. Carnosine concentrations were higher in both type I ($p < 0.05$) and type IIA fibres ($p < 0.001$) in trained horses. There was no significant difference in the carnosine concentration in type IIB fibres between untrained and trained horses ($p > 0.05$). Taurine concentration were higher in type I fibres ($p < 0.001$), and type IIA and IIB fibres ($p < 0.05$) in trained horses. Frequency distribution plots of normalized carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.5 and 4.6, respectively.

Table 4.6 Individual mean (± SD) carnosine concentrations in type I, IIA and IIB fibres of the middle gluteal muscle of 3 untrained and 3 trained thoroughbred horses, and overall mean (± SD_p) concentrations for untrained and trained horses.

Training status	Horse	Carnosine, mmol kg^{-1} DW (number of fibres)		
		Type I	Type IIA	Type IIB
Untrained	B	32.2 ± 19.2 (25)	58.9 ± 17.6 (39)	101.5 ± 44.2 (24)
	D	11.0 ± 15.4 (26)	63.9 ± 18.6 (23)	113.4 ± 23.8 (14)
	F	26.9 ± 2.1 (2)	81.7 ± 15.4 (27)	136.5 ± 33.1 (49)
	Mean ± SD_p	**23.4 ± 17.2 (53)**	**68.2 ± 17.2 (89)**	**117.1 ± 35.3 (87)**
Trained	A	25.0 ± 10.4 (41)	101.9 ± 34.9 (17)	142.4 ± 57.1 (20)
	C	30.9 ± 11.4 (30)	87.8 ± 19.5 (35)	121.6 ± 48.6 (27)
	E	35.3 ± 26.6 (8)	87.0 ± 33.8 (21)	106.3 ± 35.8 (40)
	Mean ± SD_p	**30.4 ± 13.1 (79)**	**92.2 ± 28.1 (73) ‡**	**123.4 ± 45.4 (87)**

SD_p Pooled standard deviation

‡ Significantly different to untrained $p < 0.001$

Table 4.7 Individual mean (± SD) taurine concentrations in type I, IIA and IIB fibres of the middle gluteal muscle of 3 untrained and 3 trained thoroughbred horses, and overall mean (± SD_p) concentrations for untrained and trained horses.

Training status	Horse	Taurine, mmol kg^{-1} DW (number of fibres)		
		Type I	Type IIA	Type IIB
Untrained	B	36.4 ± 11.0 (25)	12.1 ± 7.6 (39)	9.1 ± 11.9 (24)
	D	33.6 ± 17.6 (26)	10.1 ± 6.9 (23)	9.8 ± 7.1 (14)
	F	49.9 ± 15.2 (2)	13.3 ± 7.7 (27)	12.4 ± 10.3 (49)
	Mean ± SD_p	**40.0 ± 14.8 (53)**	**11.8 ± 7.5 (89)**	**10.4 ± 10.4 (87)**
Trained	A	59.6 ± 7.6 (41)	11.9 ± 6.6 (17)	9.9 ± 5.5 (20)
	C	53.2 ± 15.4 (30)	13.5 ± 8.5 (35)	10.9 ± 11.6 (27)
	E	74.6 ± 26.4 (8)	19.7 ± 12.3 (21)	20.9 ± 14.0 (40)
	Mean ± SD_p	**62.5 ± 13.6 (79) ‡**	**15.0 ± 9.4 (73) †**	**13.9 ± 11.8 (87)**

SD_p Pooled standard deviation

† Significantly different to untrained $p < 0.05$

‡ Significantly different to untrained $p < 0.001$

Figure 4.5 Frequency distribution plots of normalized carnosine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 3 untrained (n = 239) and 3 trained (n = 229) thoroughbred horses.

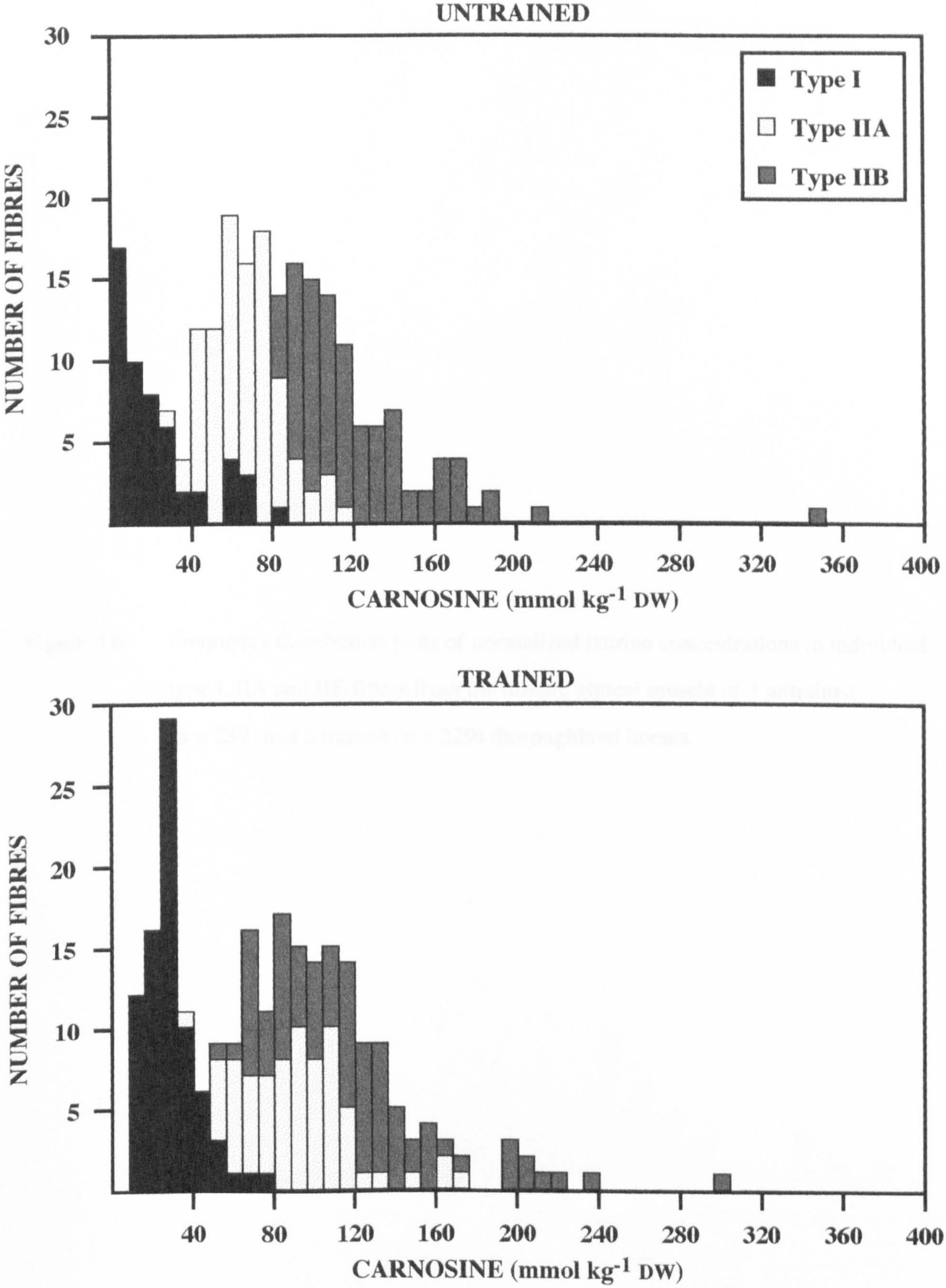
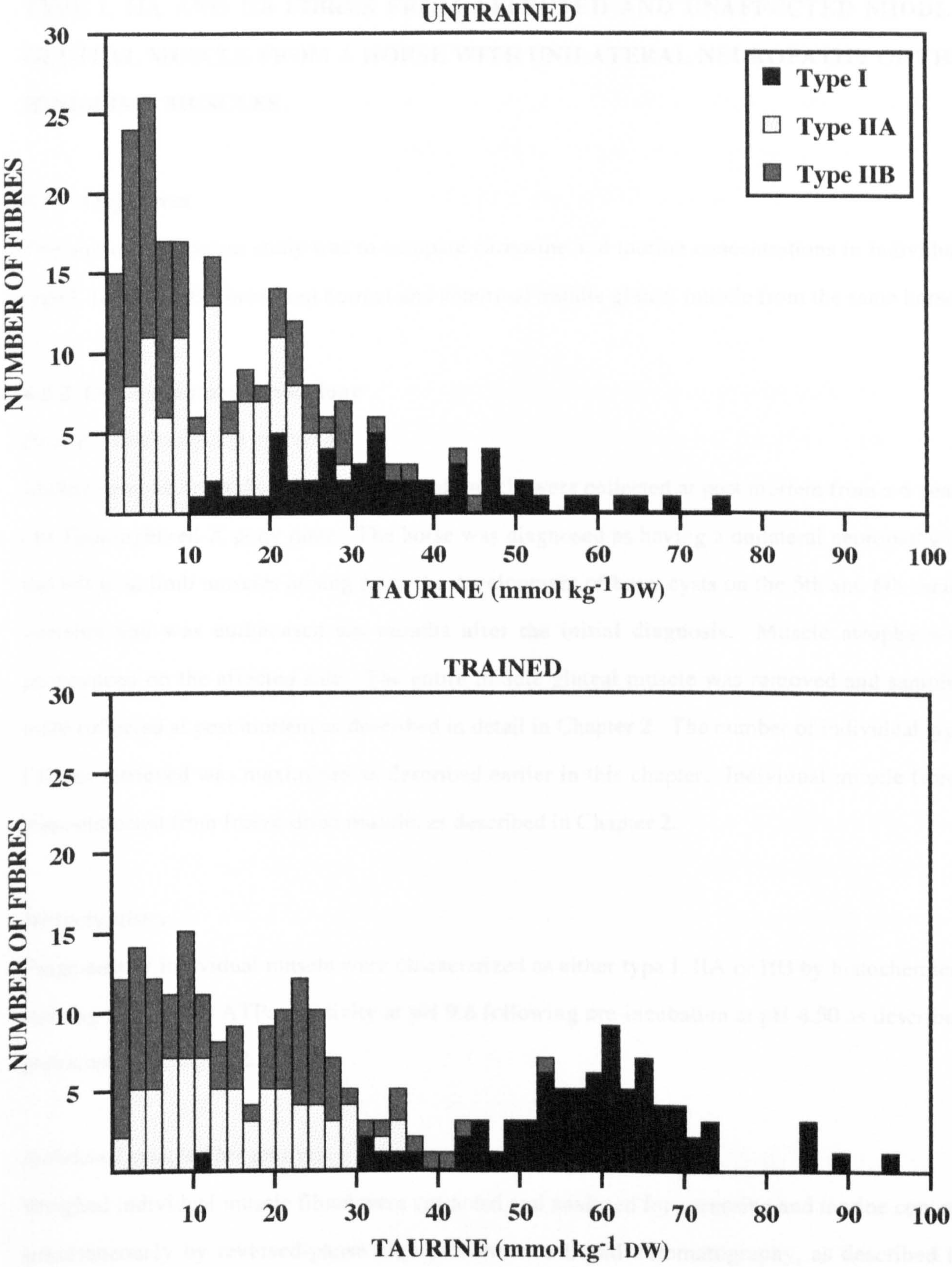

UNTRAINED
NUMBER OF FIBRES
30
25
20
15
10
5
Type I
Type IIA
Type IIB
40
80
120
160
200
240
280
320
360
400
CARNOSINE (mmol kg-1 DW)
TRAINED
NUMBER OF FIBRES
30
25
20
15
10
5
40
80
120
160
200
240
280
320
360
400
CARNOSINE (mmol kg-1 DW)

Figure 4.6 Frequency distribution plots of normalized taurine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 3 untrained (n = 239) and 3 trained (n = 229) thoroughbred horses.


UNTRAINED
NUMBER OF FIBRES
30
25
20
15
10
5
Type I
Type IIA
Type IIB
10
20
30
40
50
60
70
80
90
100
TAURINE (mmol kg-1 DW)
TRAINED
NUMBER OF FIBRES
30
25
20
15
10
5
10
20
30
40
50
60
70
80
90
100
TAURINE (mmol kg-1 DW)

4.5 STUDY D: COMPARISON OF THE CARNOSINE AND TAURINE CONTENTS IN TYPE I, IIA AND IIB FIBRES FROM AFFECTED AND UNAFFECTED MIDDLE GLUTEAL MUSCLE FROM A HORSE WITH UNILATERAL NEUROPATHY OF THE HIND-LIMB MUSCLES.

4.5.1 Objectives

The aim of the present study was to compare carnosine and taurine concentrations in individual type I, IIA and IIB fibres from normal and abnormal middle gluteal muscle from the same horse.

4.5.2 Experimental methodology

Protocol and sampling procedure

Muscle samples from the left middle gluteal muscle were collected at post mortem from a 6 year-old Thoroughbred-X pony mare. The horse was diagnosed as having a unilateral neuropathy of the left hind limb muscles arising from the development of bony cysts on the 5th and 6th sacral vertebra and was euthanased six months after the initial diagnosis. Muscle atrophy was pronounced on the affected side. The entire middle gluteal muscle was removed and samples were collected at post mortem as described in detail in Chapter 2. The number of individual type I fibres retrieved was maximized as described earlier in this chapter. Individual muscle fibres were dissected from freeze-dried muscle, as described in Chapter 2.

Histochemistry

Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously in Chapter 2.

Individual muscle fibre analysis

Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).

Statistics

Mean (± SD) values for carnosine and taurine concentrations in type I, IIA and IIB fibres, were calculated. One-factor ANOVA was used to identify significant differences in mean (± SD) values for carnosine and taurine concentrations in type I, IIA and IIB fibres between the normal and affected (neuropathic) middle gluteal muscles. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at $p < 0.05$.

4.5.3 Results

Carnosine and taurine concentrations, and taurine : carnosine ratios were determined in a total of 68 individual fibres from both normal and neuropathic middle gluteal muscles. Mean (± SD) carnosine and taurine concentrations in type I, IIA and IIB fibres are given in Table 4.8.

Carnosine and taurine concentrations in fibres from the unaffected middle gluteal muscle were similar to those found previously in normal horses. Taurine concentrations were slightly higher, particularly in type IIA and IIB fibres. This horse however, was not pure Thoroughbred and hence the higher taurine contents may been the result of inter-breed differences. As found previously in normal horses, carnosine concentrations were significantly different between type I fibres and both type IIA and type IIB fibres ($p < 0.001$). There was no significant difference between type IIA and IIB fibres ($p > 0.05$). Taurine contents were significantly different between type I fibres and both type IIA and IIB fibres ($p < 0.001$). There was no significant difference between type IIA and IIB fibres ($p > 0.05$).

The taurine concentration in type I fibres from the neuropathic middle gluteal muscle was significantly lower than in fibres from the unaffected middle gluteal muscle ($p < 0.001$). Taurine concentrations in type IIA and IIB fibres were not significantly different between the neuropathic and normal middle gluteal muscles ($p < 0.05$). The carnosine concentration in type I fibres from the neuropathic middle gluteal muscle was not significantly different to the unaffected muscle ($p < 0.05$). The carnosine concentration in type IIA fibres from the

neuropathic middle gluteal muscle was lower than the corresponding value in the normal middle gluteal. The difference however, was not significant ($p > 0.05$). The carnosine concentration in type IIB fibres from the neuropathic middle gluteal was significantly lower than in type IIB fibres from the unaffected middle gluteal ($p < 0.01$).

Frequency distribution plots of carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.7 and 4.8, respectively.

Table 4.8 Carnosine and taurine concentrations in unaffected and affected (neuropathic) muscle fibres.

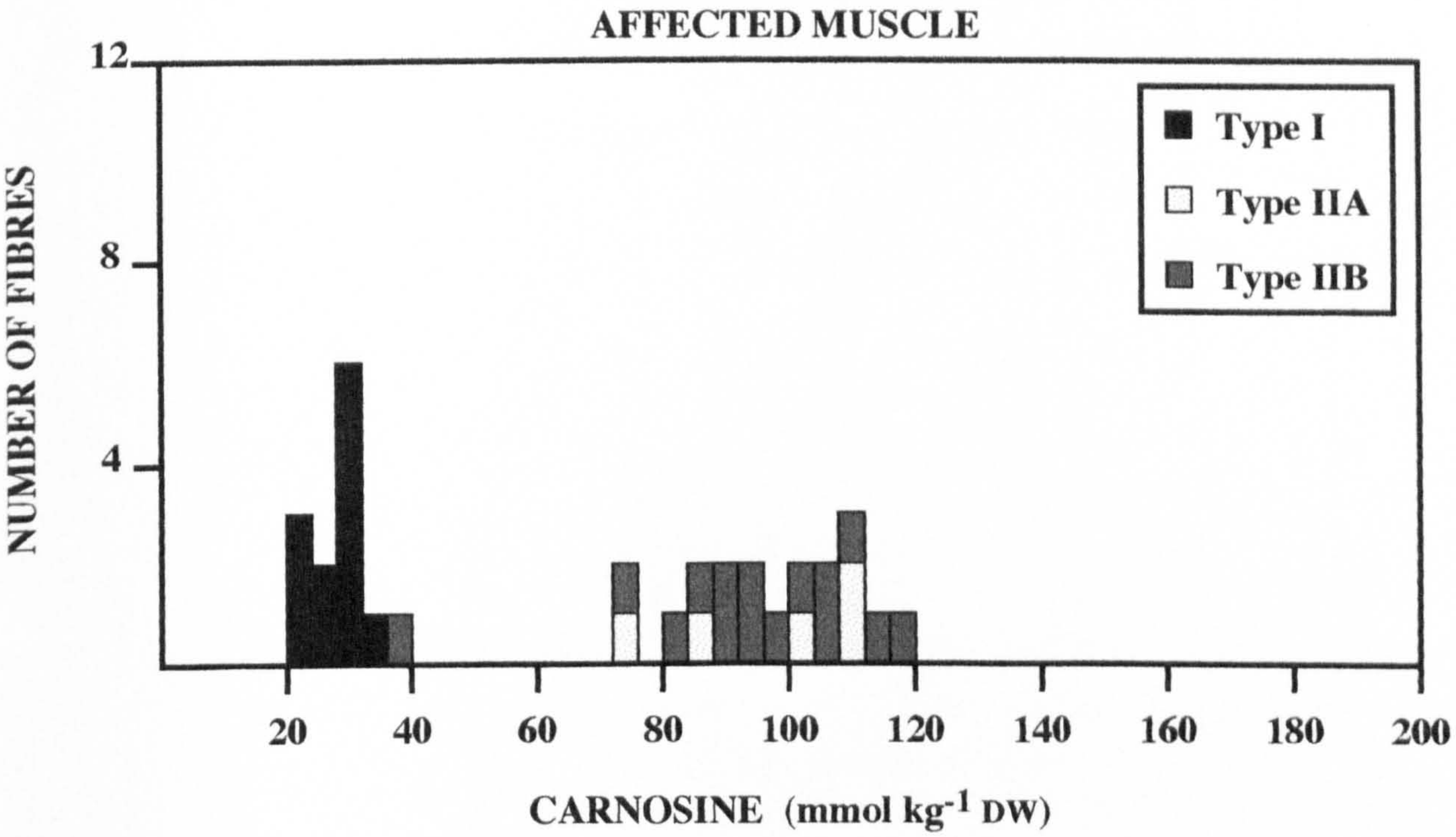
Fibre type	Muscle	n	Carnosine	Taurine
			mmol kg^{-1} DW	mmol kg^{-1} DW
Type I	Unaffected	13	24.5 ± 5.4	73.2 ± 9.4
	Affected	14	27.6 ± 3.1	39.9 ± 4.3‡
Type IIA	Unaffected	9	100.8 ± 23.8	9.7 ± 2.2
	Affected	5	96.9 ± 16.0	11.0 ± 1.2
Type IIB	Unaffected	12	122.6 ± 30.0	11.9 ± 2.7
	Affected	15	93.4 ± 20.1†	10.1 ± 2.1

n = Number of fibres

† Significantly different to unaffected fibres $p < 0.01$

‡ Significantly different to unaffected fibres $p < 0.001$

Figure 4.7 Carnosine distribution in unaffected and affected (neuropathic) type I, IIA and IIB muscle fibres.


AFFECTED MUSCLE
NUMBER OF FIBRES
12
8
4
Type I
Type IIA
Type IIB
20
40
60
80
100
120
140
160
180
200
CARNOSINE (mmol kg-1 DW)

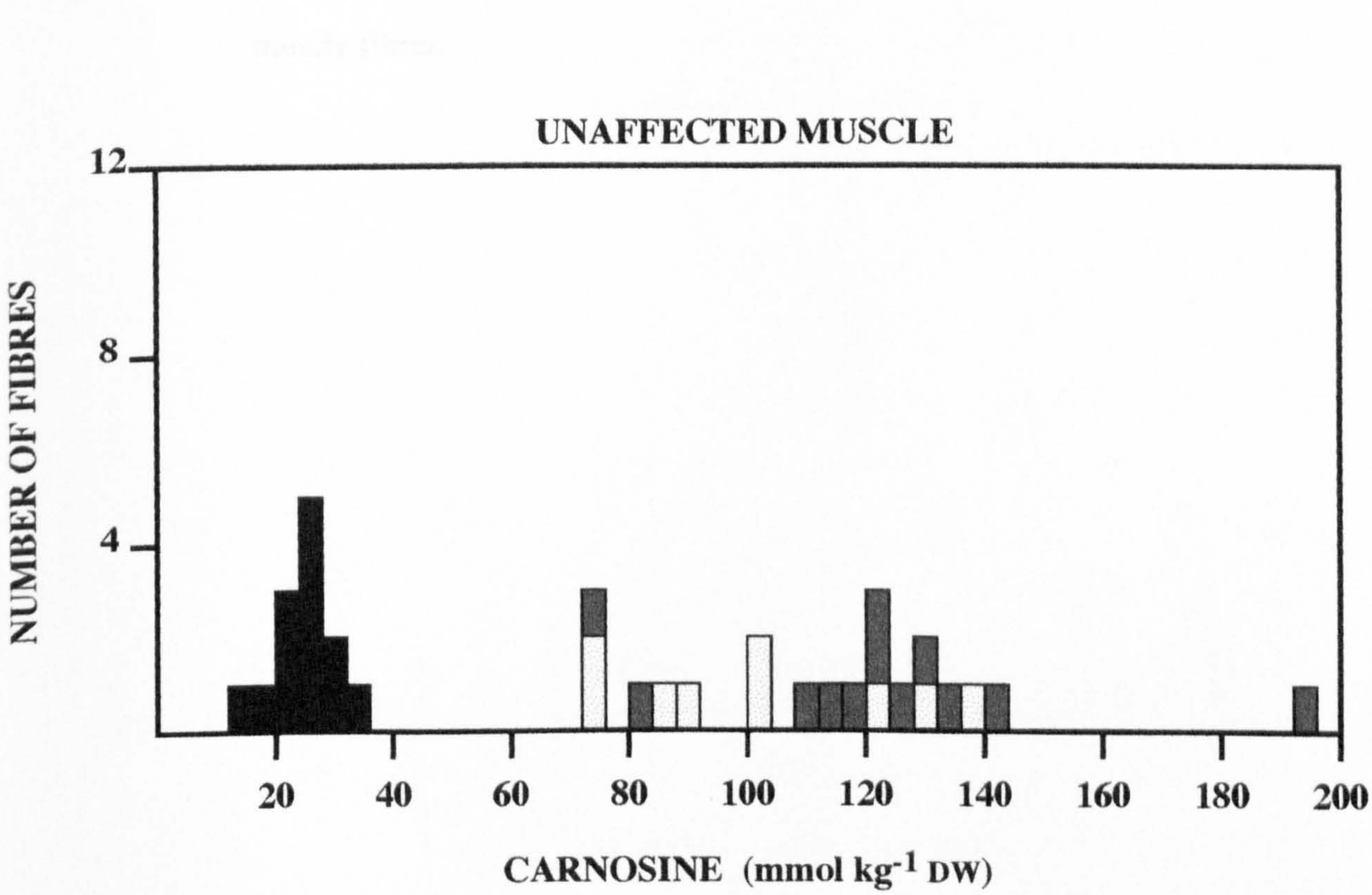
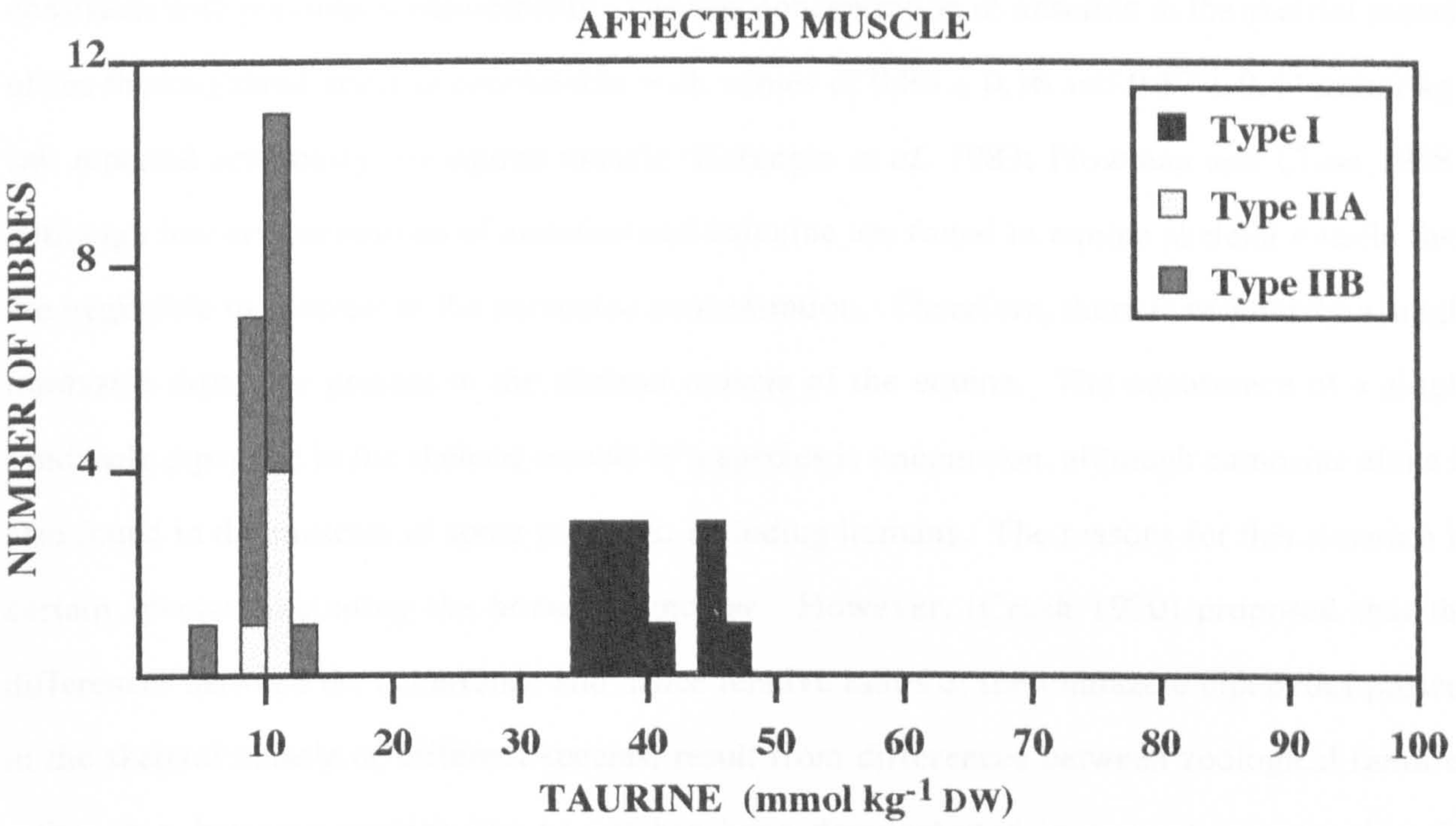
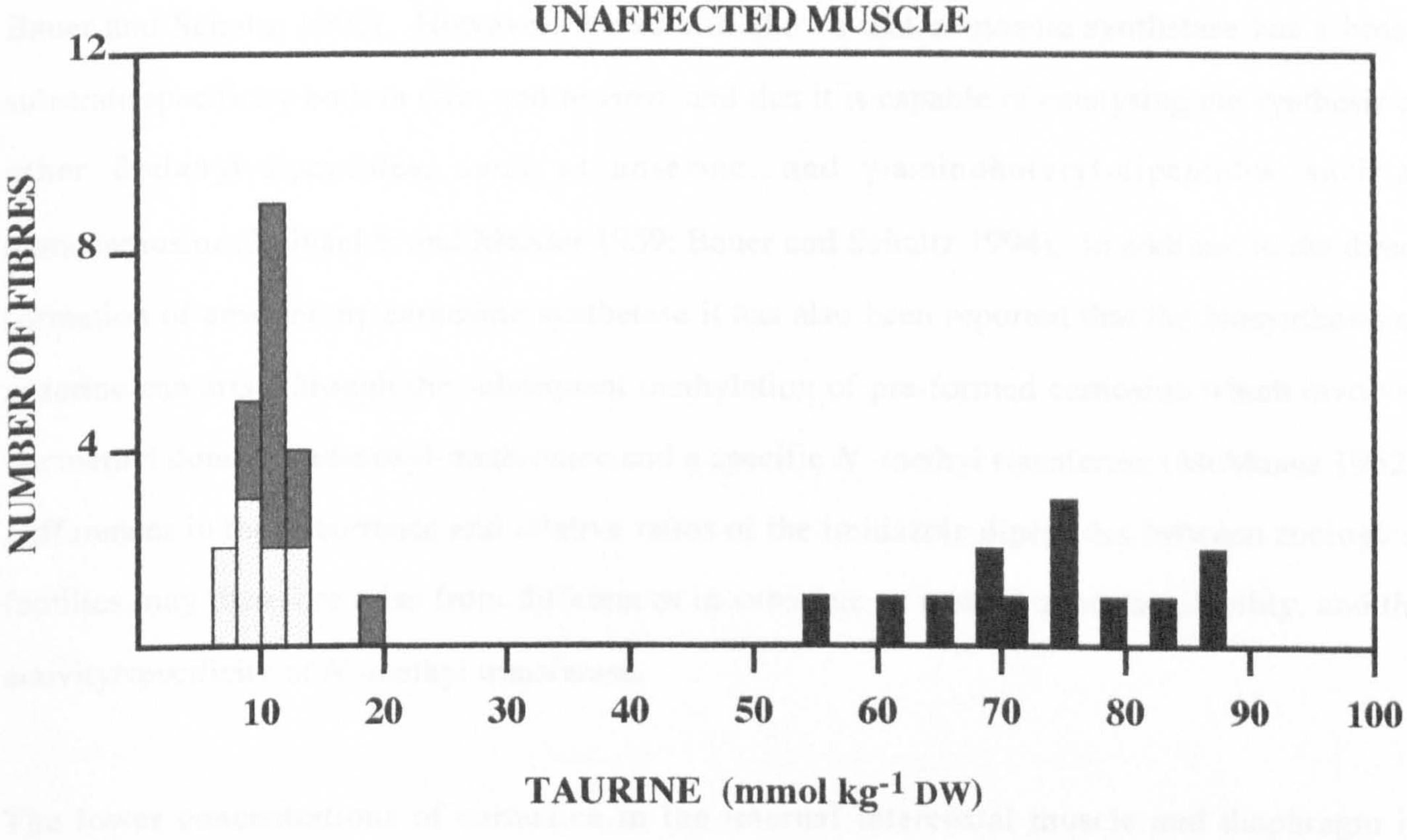

UNAFFECTED MUSCLE
NUMBER OF FIBRES
12
8
4
20
40
60
80
100
120
140
160
180
200
CARNOSINE (mmol kg-1 DW)

Figure 4.8 Taurine distribution in unaffected and affected (neuropathic) type I, IIA and IIB muscle fibres.


AFFECTED MUSCLE
12
8
4
NUMBER OF FIBRES
Type I
Type IIA
Type IIB
10
20
30
40
50
60
70
80
90
100
TAURINE (mmol kg-1 DW)


UNAFFECTED MUSCLE
12
8
4
NUMBER OF FIBRES
10
20
30
40
50
60
70
80
90
100
TAURINE (mmol kg-1 DW)

4.6 DISCUSSION

The mean carnosine concentration in the middle gluteal muscle measured in Study A is consistent with previous measurements. The low concentration of anserine in the skeletal muscle of the thoroughbred horse is comparable with values of 0.80 ± 0.16 and 0.37 ± 0.44 mmol kg^{-1} DW reported previously for equine muscle (Carnegie *et al.* 1983; Plowman and Close 1988). Although low concentrations of anserine and balenine are found in equine skeletal muscle they are negligible in contrast to the carnosine concentration. Therefore, there is essentially a single imidazole dipeptide present in the skeletal muscle of the equine. The occurrence of a single imidazole dipeptide in the skeletal muscle of a species is uncommon, although carnosine alone is also found in the muscles of some primates, including humans. The reasons for this situation in certain species, including the horse, is unclear. However, (Crush 1970) proposed that the differences between the occurrence and hence relative ratios of the imidazole dipeptides present in the skeletal muscle of different species, result from differences between zoological families rather than between species *per se*. It has been demonstrated in several species, that the biosynthesis of carnosine occurs directly from its constituent amino acids, histidine and ß-alanine, and is catalysed by the enzyme carnosine synthetase (Kalyanker and Meister 1959; Bauer and Schultz 1994). However, it was also shown that carnosine synthetase has a broad substrate specificity both *in vivo* and *in vitro* and that it is capable of catalysing the synthesis of other ß-alanyl-dipeptides, such as anserine, and γ-aminobutyryl-dipeptides, such as homocarnosine (Kalyanker and Meister 1959; Bauer and Schultz 1994). In addition to the direct formation of anserine by carnosine synthetase it has also been reported that the biosynthesis of anserine can arise through the subsequent methylation of pre-formed carnosine which involves the methyl donor *S* -adenosyl-methionine and a specific *N* -methyl transferase (McManus 1962). Differences in the occurrence and relative ratios of the imidazole dipeptides between zoological families may therefore arise from differences in substrate or methyl donor availability, and the activity/specificity of *N* -methyl transferase.

The lower concentrations of carnosine in the internal intercostal muscle and diaphragm in contrast to the middle gluteal muscle probably reflect the different functions of the former. The

middle gluteal muscle is essentially a locomotory skeletal muscle which although involved in maintaining posture and locomotion during low-intensity exercise, its function is of greater importance during high-intensity exercise, such as sprinting and jumping. Consequently there is a preponderance of fast-contracting type II fibres in this muscle. In contrast, the internal intercostal muscles and the diaphragm are involved in ventilation, a process which requires a lower rate of muscle contraction. As such they are likely to have a lesser proportion of type II fibres resulting in a lower overall glycolytic capacity, therefore lower lactic acid production and consequently a reduced need for H^+ ion buffering. The two to three-fold lower carnosine concentrations in these muscles relative to the middle gluteal is consistent with their reduced demand for H^+ ion buffering. The carnosine concentration in diaphragm is similar to the estimated value for type I skeletal muscle fibres which suggests a predominance of these fibres in this muscle, although differences in the carnosine concentration within a given fibre type between different muscles is also possible.

Carnosine concentrations measured in cardiac muscle (myocardium), smooth muscle (small intestine, colon, stomach and rectum) and other non-muscle tissues were fifty-fold or more lower than those found in the middle gluteal muscle. Such low concentrations of carnosine provide a minimal H^+ ion buffering capacity , therefore its presence in these tissues may be explained by some of the other ascribed physiological functions. Carnosine concentrations in equine myocardium are similar to values reported in the cardiac muscle of other vertebrate species (Sobue *et al.* 1975; O'Dowd *et al.* 1988). Total concentrations of acetylated imidazole dipeptides in cardiac muscle from rat, guinea pig and frog were greater than 40 mmol kg^{-1} DW (O'Dowd *et al.* 1988) and it is therefore possible that the presence of the non-acetylated forms of the dipeptides, including carnosine, arise from metabolism of the acetylated forms, and that carnosine *per se* has no physiological role in cardiac muscle.

The specific activity attributable to tissue carnosinase was not discriminated from the activity of non-specific dipeptidase in the assay employed. However, the total activity for carnosine degradation is of greater physiological relevance. The mean total carnosinase and non-specific

dipeptidase activity was higher in the small intestine, kidney, spleen and lung of the thoroughbred horse than in the other tissues examined. The activity in the small intestine was less than the value of 0.36 μmol g^{-1} min^{-1} WW (86 μmol g^{-1} h^{-1} DW) reported in the rat (Tamaki *et al.* 1985), and considerably lower than the value of 8.8 μmol g^{-1} min^{-1} WW (2112 μmol g^{-1} h^{-1} DW) reported in the human (Sadikali *et al.* 1975). As the site of tissue sampling was not standardized in the equine small intestine the large standard deviation associated with the tissue carnosinase and non-specific dipeptidase activity is probably due to a decrease in the activity of these enzymes towards the ileocaecal valve. Such a distribution has previously been described in the small intestine of the rat (Tamaki *et al.* 1985). The lower activity in equine small intestine probably reflects the dietary absence of carnosine in the equine, in contrast to the carnosine containing diets of the other two species. The mean total carnosinase and non-specific dipeptidase activity in the equine kidney was lower than that reported in the rat 4.76 μmol g^{-1} min^{-1} WW (1142 μmol g^{-1} h^{-1} DW) (Tamaki *et al.* 1985). The relatively high values found in the spleen and lung of the horse are also consistent with the tissue distribution in the rat.

Re-determination of tissue carnosinase and non-specific dipeptidase activities in some tissues was performed in the presence of 0.2 μM bestatin, an inhibitor of non-specific dipeptidase but not tissue carnosinase. Although this does not completely inhibit non-specific dipeptidase, it enabled an estimate to be made of the activity attributable to tissue carnosinase alone. This indicated that the true tissue carnosinase activity in the kidney, spleen and small intestine was only 36.7, 15.0 and 14.6% of the total activity, respectively. Enzyme activities measured *in vitro* do not necessarily represent accurately the true *in vivo* value. However, the apparent low overall tissue carnosinase activities, and absence of carnosinase activity in skeletal muscle, in the thoroughbred horse are probably indicative of a need to limit carnosine catabolism in order to maintain the high muscle carnosine concentrations required for optimum H^+ ion buffering.

The studies described in this chapter are the first to measure the concentrations of carnosine and taurine directly in individual skeletal muscle fibres. Earlier studies relied upon indirect estimates derived from multiple linear regression analysis of data relating muscle carnosine and taurine

concentrations to type I, IIA and IIB % FSA in 'large' mixed fibre muscle samples (Sewell *et al.* 1990; Sewell *et al.* 1992; Dunnett *et al.* 1992). The present values for carnosine concentrations in type I, IIA and IIB fibres are in close agreement with the estimates of (Sewell *et al.* 1990). These earlier studies determined carnosine concentrations in muscle obtained from horses of differing ages and states of training. When the present measurements are compared with these previous estimates the agreement is not as close with respect to type I and type IIB fibres although the same trend is apparent, i.e. the highest carnosine concentration occurring in type IIB fibres and the lowest in type I fibres, is apparent. This disparity may be the result of deficiencies in the earlier mathematical model, or more probably arose from the exclusive use of young (two year-old) highly trained horses. These horses underwent high-intensity training and racing and hence would have an increased requirement for intra-muscular buffering capacity.

The values for taurine concentrations in type I, IIA and IIB fibres in study B are similar to previous estimates for older horses sampled at post mortem (Dunnett *et al.* 1992), and the general finding is identical, i.e. taurine is almost exclusively localized within type I fibres. A poorer agreement with estimates derived from percutaneous muscle biopsy samples may arise for those reasons mentioned previously; namely the exclusive sampling of young intensely trained animals (Dunnett *et al.* 1992). A similar distribution of carnosine and taurine has also been observed in type I, IIA and IIB fibres in the camel (Dunnett and Harris 1995c).

Previous experimental models have predicted differences between the mean carnosine concentrations of type IIA and IIB fibres of 20 and 95 mmol kg^{-1} DW, respectively (Sewell *et al.* 1990; Sewell *et al.* 1992). The current data however, shows a difference of only 9.6 mmol kg^{-1} DW. This discrepancy may arise from limitations within the fibre characterization procedure where uncertainty may arise owing to variations in fibre thickness. In contrast fewer problems of classification are encountered when staining sections of uniform thickness and where the assessor is presented with a field of mixed fibres to be viewed simultaneously. The difference in carnosine concentrations between type I and type II muscle fibres is consistent with their individual metabolic characteristics (Snow 1982). During low intensity exercise type I fibres are

predominantly recruited (Lindholm *et al.* 1974; Snow *et al.* 1982), H^+ ion production is minimal and a lower buffering capacity is required (Sewell *et al.* 1990; Sewell *et al.* 1992). During high intensity exercise type II fibres are recruited (Lindholm *et al.* 1974; Snow *et al.* 1982) and the high rate of glycolysis requires a large *in situ* H^+ ion buffering capacity (Sewell *et al.* 1990; Sewell *et al.* 1992). Some buffering capacity from carnosine within type I fibres may be necessary owing to probable diffusion of H^+ ions into these fibres during periods of anaerobic exercise. It has also been proposed that carnosine functions as an antioxidant *in vivo* and that it is able to protect membrane lipids against damage from reactive oxygen species (Esterbauer *et al.* 1986; Boldyrev *et al.* 1988; Kohen *et al.* 1988). This provides an alternative explanation for the presence carnosine in the oxidative type I muscle fibres.

Without a clearer understanding of the functions of taurine within skeletal muscle *per se* it is difficult to establish a rationale for the much higher taurine concentrations in type I fibres. The presence of such high concentrations of taurine within these fibres suggest some significant physiological or biochemical role. Taurine may possibly compensate for the lower concentrations of carnosine by maintaining the osmolarity within type I muscle cells. It has also been proposed that taurine functions as an antioxidant and exerts a protective effect against reactive oxygen species *in vivo* (Franconi *et al.* 1985) and through reaction with malondialdehyde (Ogasawara *et al.* 1993). This may be a further explanation for the presence of high a taurine concentration in type I muscle fibres, a highly oxidative tissue.

Changes in the carnosine and taurine concentrations within the different fibre types, as a consequence of training, as described in Study C, are likely to be influenced by the relative contributions of aerobic and anaerobic pathways to energy production during exercise as part of the training programme. Bump *et al.* (1989) reported a decrease in muscle carnosine concentration in Quarterhorses during training. However, the 7 week training protocol was only of moderate intensity, as shown by the relatively low peak mean plasma lactate concentration of 87.6 mg 100 ml^{-1} (9.7 m*M*). A training protocol with a high anaerobic component will result in large increases in muscle lactate and associated H^+ ion concentrations. As a result of exposure

of the musculature to repeated periods of acidosis the significant increase in the carnosine concentration in IIA muscle fibres in the middle gluteal muscle suggests an adaptive response within the integrated system for the maintenance of intra-muscular pH homeostasis. A previously reported increase in β_m in the middle gluteal muscle of the horse during high-intensity training (McCutcheon *et al.* 1987; Sinha *et al.* 1991), could be explained by an increase in the muscle carnosine concentration, such as that observed in Study C. It is possible that the observed increase in the carnosine concentration in type IIA fibres only, is indicative of greater 'trainability' in these mixed oxidative-glycolytic fibres.

Without a clear understanding of the precise function(s) of taurine in skeletal muscle it is difficult to explain the observed increase in taurine concentration in all three fibre types as a result of training. It has been proposed that taurine functions as an antioxidant *in vivo* (Franconi *et al.* 1985; Ogasawara *et al.* 1993), hence the increase in muscle taurine concentration may be a consequence of the aerobic component of the training protocol.

Unilateral ischemic denervation of gastrocnemius muscle in experimental rats has been shown to cause a significant decline ($p < 0.01$) in the muscle carnosine concentration over a period of three weeks (Tamaki *et al.* 1976). A similar study involving the denervation of the soleus, plantaris and gastrocnemius muscles in experimental rats, showed a non-significant decrease in the carnosine content of the soleus and plantaris muscles and a significant increase in the carnosine concentration ($p < 0.05$) in the gastrocnemius muscle (Turnisky and Long 1990). However, these muscles were analysed only three days after denervation, in contrast to three weeks after in the study of Tamaki *et al* (1976). The present results for changes in muscle carnosine concentration during denervation are consistent with the evidence from these two earlier studies on whole muscles.

CHAPTER 5

EQUINE PLASMA CARNOSINE CONCENTRATION AND CARNOSINASE ACTIVITY: NORMAL VALUES, DAILY VARIATION, AND THE EFFECTS OF EXERCISE AND MUSCLE DAMAGE

5.1 INTRODUCTION

Plasma carnosine concentrations appear to have been measured in very few species. Not surprisingly most measurements have been made in human plasma. Carnosine is present in the plasma of human neonates at concentrations of less than 1.2 mg l^{-1} (5.3 μM) in pre-term infants on a low protein diet and 2.0 ± 1.6 mg l^{-1} (8.8 ± 7.1 μM) in pre-term infants on a high protein diet. Lower concentrations of 0.7 ± 1.7 mg l^{-1} (3.1 ± 7.5 μM) were found in older full-term infants (Valman *et al.* 1971). Carnosine is however, absent from the plasma of normal adults. This age related disappearance of carnosine appears to be correlated with an increase in serum carnosinase activity. Serum carnosinase activity in young children (less than 1 year old) is 0.14 ± 0.07 and 0.22 ± 0.15 μmol ml^{-1} h^{-1} for males and females, respectively, in contrast to adult values of 1.85 ± 0.59 - 9.66 ± 0.42 μmol ml^{-1} h^{-1} (Bando *et al.* 1986; Bando *et al.* 1984; Wassif *et al.* 1994). Relatively high plasma carnosine concentrations (hypercarnosinemia) have been reported in cases of human serum carnosinase deficiency (Perry *et al.* 1968; Perry *et al.* 1967).

Carnosine has been detected in the plasma and other blood constituents of chicks, rats and rabbits. Chick plasma and rabbit serum were reported to contain 27 ± 3 μM and 1.9 - 7.7 μM carnosine, whereas only trace amounts were found in rat plasma (Seely and Marshall 1981; Kurisaki and Hiraiwa 1988). However, chick erythrocytes contained large amounts of carnosine (2510 ± 70 nmol g^{-1} cells), a value comparable with chick skeletal muscle. Lower quantities were present in rabbit reticulocytes (105 ± 11 nmol g^{-1} cells) and erythrocytes (18 ± 2 nmol g^{-1} cells), and only trace amounts in rat erythrocytes (< 5 nmol g^{-1} cells) (Seely and Marshall 1981). The occurrence of carnosine in frog erythrocytes has also been reported (Balgooy *et al.* 1974). The high concentration of carnosine in chick erythrocytes was associated with the presence of carnosine synthetase activity (Ng and Marshall 1976b). Carnosine has also been detected in the blood of some species of fish, such as trace amounts in Rainbow trout blood (Abe 1991) and a concentration of 0.67 ± 0.17 μmol g^{-1} in tuna blood (Abe *et al.* 1986). The only previously reported measurement of carnosine concentration in equine plasma recorded values of between 1.7 and 5.2 μM (McLean *et al.* 1987).

With the exception of diet (i.e. the consumption of meat and associated foodstuffs) no previous investigations appeared to consider other possible factors, such as diurnal variation and exercise, which may influence the concentration of carnosine in plasma. Owing to the extremely high concentration of carnosine in skeletal muscle relative to plasma it is possible that significant increases in the plasma concentration may arise as a consequence of muscle damage. Large increases in both plasma carnosine and anserine concentrations in the rat following traumatic shock induced muscle damage have been reported (Kurisaki and Hiraiwa 1988).

5.2 STUDY A: NORMAL PLASMA CARNOSINE CONCENTRATION AND CARNOSINASE ACTIVITY IN THE THOROUGHBRED HORSE, AND THE INFLUENCE OF AGE AND GENDER.

5.2.1 Objectives

The objectives of this study were to establish the normal range in plasma carnosine concentration and plasma carnosinase activity in normal thoroughbred horses and to investigate the possible influence of age, gender, and plasma histidine and ß-alanine concentrations.

5.2.2 Experimental methodology

Protocol and sampling procedure

Single heparinized venous blood samples (5 ml) were obtained from 112 thoroughbred horses in training (26 males, 30 females, 25 geldings), yearlings (13 males, 13 females) and foals (2 males, 3 females) from four racing yards and one stud farm in the Newmarket area. All blood samples were collected between 15.00 and 17.00 h.

Analytical methods

Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3 (Dunnett and Harris 1992). Plasma ß-alanine concentrations were determined as described in Chapter 2. Plasma carnosinase activity was determined using a modification of the method of Bando *et al.* (1984), as described in Chapter 2.

Statistical analysis

A two factor analysis of variance (ANOVA) was used to identify significant effects of age and gender. Significance was declared at $p < 0.05$. In the instance where significance was detected a multiple comparison test, Fisher's Protected Least Significant Different (PLSD), was applied.

5.2.3 Results

Preliminary measurements of plasma carnosine, histidine, anserine and 1-methylhistidine concentrations in a small group of experimental horses (n = 8) have been reported (Dunnett and Harris 1992). Anserine was absent from equine plasma and 1-methylhistidine was present at 4.64 ± 0.97 μM. No further determinations of 1-methylhistidine concentrations were made. Carnosine, histidine and ß-alanine concentrations in plasma were measured in a further 104 Thoroughbreds. Anserine and ß-alanine were absent from the plasma of all horses (n = 112). The ranges in plasma carnosine and histidine concentrations in thoroughbred horses of different ages and gender are shown in Figures 5.1 and 5.2, respectively. Mean plasma carnosine and histidine concentrations for these horses are given in Tables 5.1 and 5.2, respectively. The mean ($\pm$ SD) carnosine and histidine concentrations in all horses were 10.2 ± 3.8 μM and 51.4 ± 10.1 μM, respectively. There was no significant difference in either plasma carnosine or histidine concentrations between males, females and geldings at any age ($p > 0.05$), with the exception of a significant difference in plasma histidine concentration between 3 year-old geldings and males ($p < 0.05$). There was a significant effect of age on both plasma carnosine and histidine concentrations which was more marked with respect to the former. Plasma carnosine concentrations in both foals and yearlings were approximately half those found in older horses ($p < 0.001$). Plasma histidine concentrations were significantly lower in foals and yearlings compared to older horses ($p < 0.05$). No significant differences in plasma carnosine and histidine concentrations were evident between older horses. The range in plasma carnosine concentrations in yearlings (3.9 - 8.7 μM) was also much narrower than in older horses (e.g. 6.5 - 17.0 and 8.2 - 21.0 μM in 2 and 3 year-olds, respectively). For horses of 2 years and older there was no significant correlation between plasma carnosine and histidine concentrations (r =

0.173, $p > 0.05$), however, a weak but significant correlation was found in foals and yearlings (r = 0.498, $p < 0.005$), as shown in Figure 5.3.

Plasma carnosinase activity was absent in samples from all horses. The plasma carnosinase assay was validated by making comparative measurements on human plasma (n = 5 in duplicate). The mean (± SD) activity obtained (2.08 ± 1.48 μmol ml^{-1} h^{-1}) was consistent with serum values reported in the literature (1.85 ± 0.59 μmol ml^{-1} h^{-1}) (Bando *et al.* 1984).

Figure 5.1 Range in plasma carnosine concentrations at different ages in male, female and gelded thoroughbred horses (n = 112).

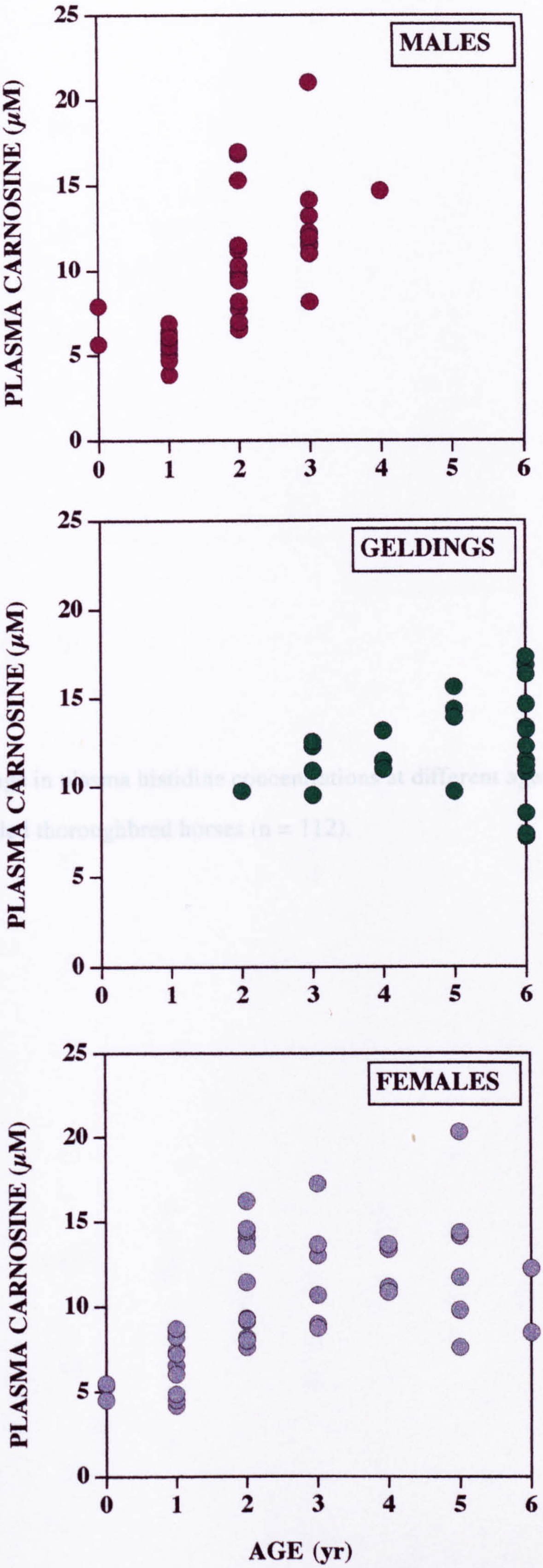
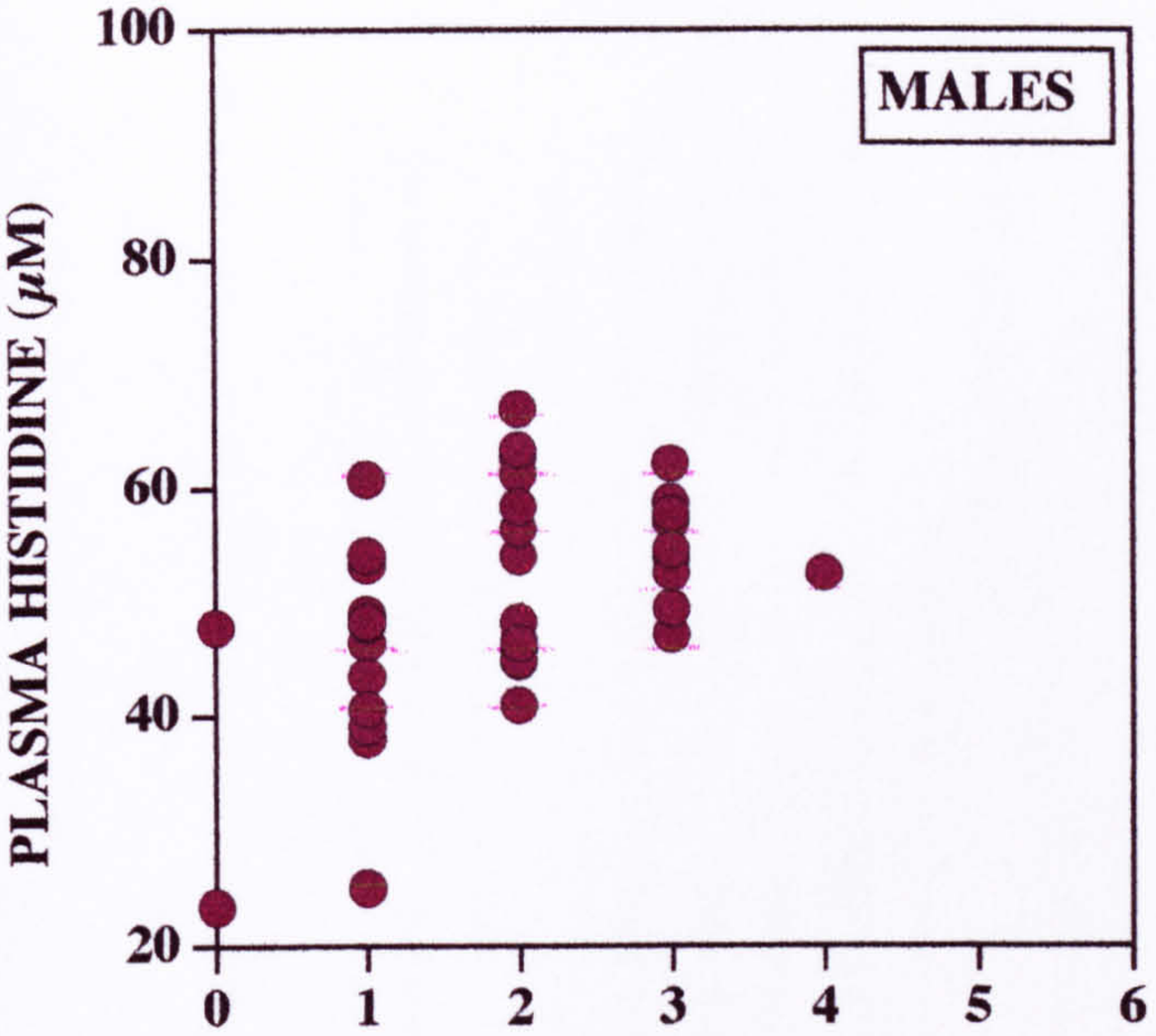
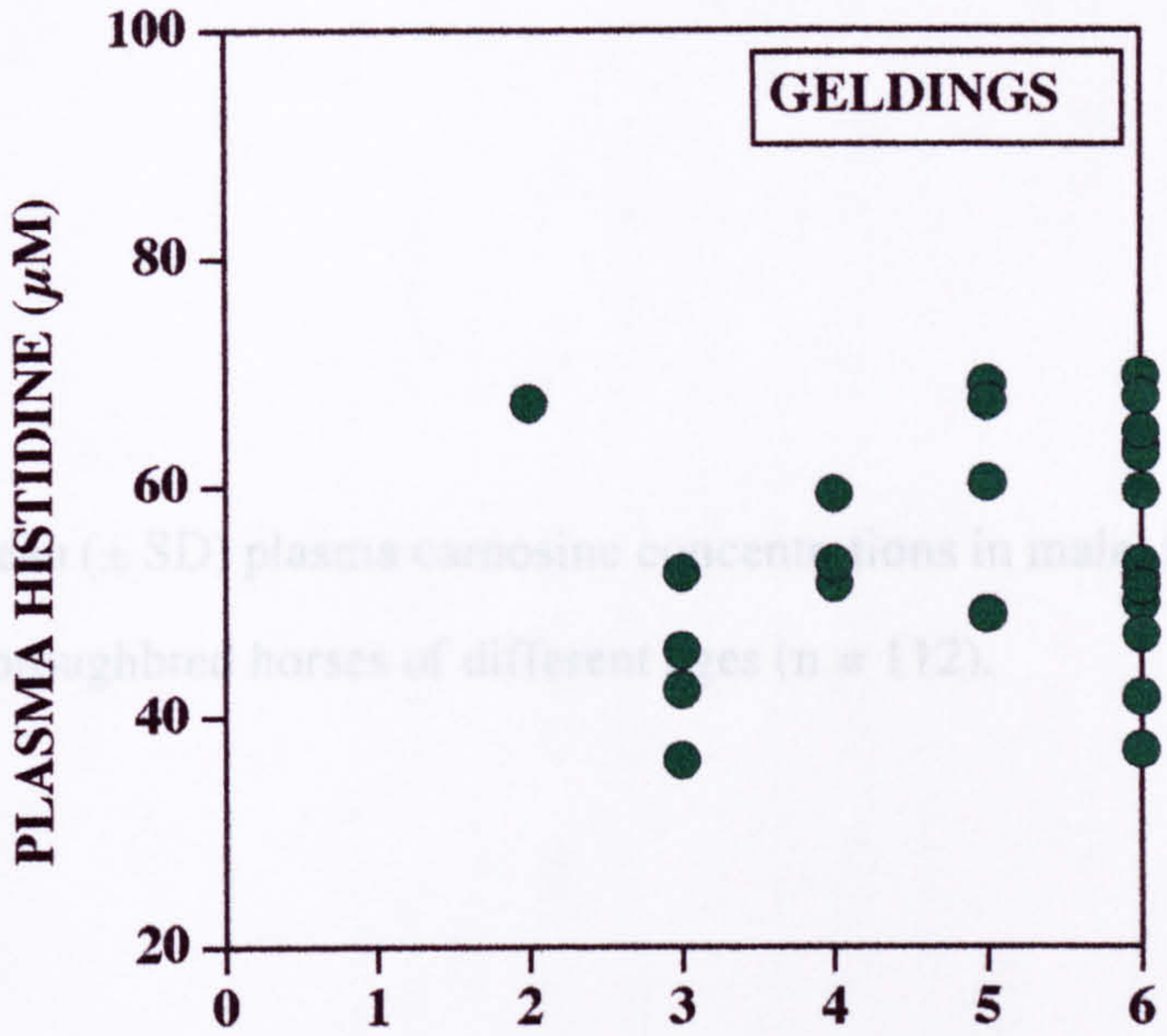

MALES
GELDINGS
FEMALES
PLASMA CARNOSINE (μM)
0
5
10
15
20
25
0
1
2
3
4
5
6
AGE (yr)

Figure 5.2 Range in plasma histidine concentrations at different ages in male, female and gelded thoroughbred horses (n = 112).


MALES
PLASMA HISTIDINE (μM)
100
80
60
40
20
0
1
2
3
4
5
6


GELDINGS
PLASMA HISTIDINE (μM)
100
80
60
40
20
0
1
2
3
4
5
6

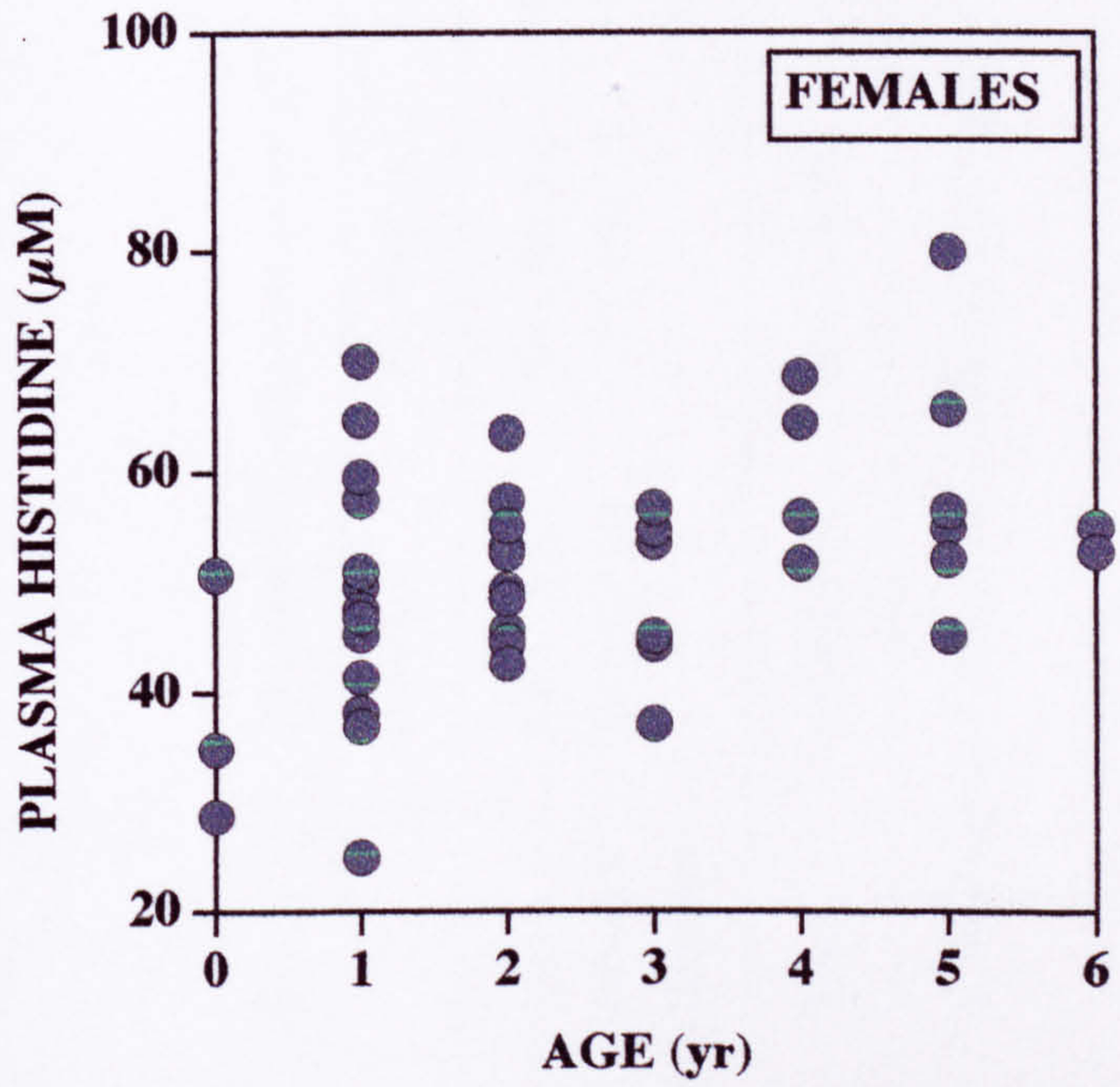

FEMALES
PLASMA HISTIDINE (μM)
100
80
60
40
20
0
1
2
3
4
5
6
AGE (yr)

Table 5.1 Mean (± SD) plasma carnosine concentrations in male, female and gelded thoroughbred horses of different ages (n = 112).

Gender	Plasma carnosine concentration (μM)							
	Foals (n)	Yearlings (n)	2 yr-olds (n)	3 yr-olds (n)	4 yr-olds (n)	5 yr-olds (n)	6 yr-olds (n)	All horses (n)
Males	6.8 ± 1.6 (2)	5.6 ± 0.8 (13)	10.3 ± 3.4 (16)	12.8 ± 3.5 (9)	14.1 (1)	-	-	9.3 ± 4.0 (41)
Females	5.1 ± 0.5 (3)	6.5 ± 1.6 (13)	11.3 ± 3.1 (12)	12.1 ± 3.2 (6)	12.3 ± 1.5 (4)	13.0 ± 4.4 (6)	10.4 ± 2.7 (2)	9.9 ± 3.8 (46)
Geldings	-	-	9.8 (1)	11.3 ± 1.4 (4)	11.9 ± 1.1 (3)	13.4 ± 2.6 (4)	12.4 ± 3.4 (13)	12.2 ± 2.8 (25)
Total	5.8 ± 1.3 (5)	6.0 ± 1.3 (26)	10.7 ± 3.2† (29)	12.2 ± 3.0† (19)	12.4 ± 1.5† (8)	13.2 ± 3.6† (10)	12.1 ± 3.3† (15)	10.2 ± 3.8† (112)

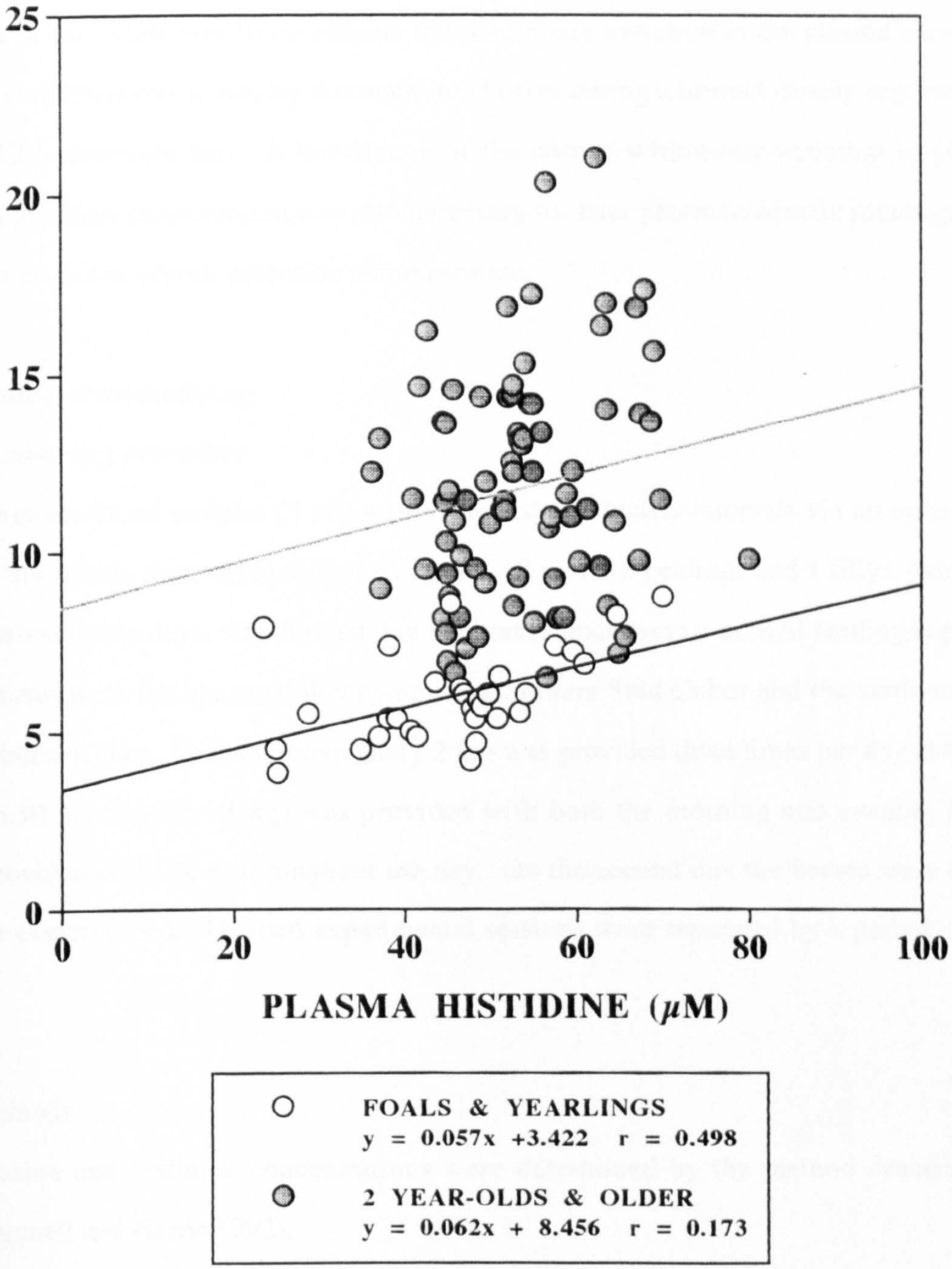
† significantly different from foals and yearlings ($p < 0.001$)

Table 5.2 Mean (± SD) plasma histidine concentrations in male, female and gelded thoroughbred horses of different ages (n = 112).

Gender	Plasma histidine concentration (μM)							
	Foals (n)	Yearlings (n)	2 yr-olds (n)	3 yr-olds (n)	4 yr-olds (n)	5 yr-olds (n)	6 yr-olds (n)	All horses (n)
Males	35.6 ± 17.2 (2)	45.7 ± 8.9 (13)	51.9 ± 8.4 (16)	54.8 ± 4.7 (9)	52.6 (1)	-	-	49.8 ± 9.2 (41)
Females	38.1 ± 11.4 (3)	48.7 ± 12.1 (13)	50.1 ± 6.4 (12)	48.6 ± 7.6 (6)	60.2 ± 7.7 (4)	59.0 ± 12.2 (6)	53.7 ± 1.6 (2)	50.9 ± 10.6 (46)
Geldings	-	-	67.3 (1)	44.2 ± 6.8 (4)	54.7 ± 4.1 (3)	61.4 ± 9.2 (4)	55.9 ± 10.2 (13)	55.0 ± 10.1 (25)
Total	37.1 ± 11.8 (5)	47.2 ± 10.6 (26)	51.7 ± 8.0† (29)	50.6 ± 7.3 (19)	57.2 ± 6.4† (8)	59.9 ± 10.6† (10)	55.2 ± 9.5† (15)	51.4 ± 10.1 (112)

† significantly different from foals and yearlings ($p < 0.05$)

Figure 5.3 Correlation between plasma carnosine and histidine concentrations in thoroughbred horses (n = 112).


25
20
15
10
5
0
0
20
40
60
80
100
PLASMA CARNOSINE (μM)
PLASMA HISTIDINE (μM)
FOALS & YEARLINGS
y = 0.057x +3.422 r = 0.498
2 YEAR-OLDS & OLDER
y = 0.062x + 8.456 r = 0.173

5.3 STUDY B: VARIATION IN PLASMA CARNOSINE AND HISTIDINE CONCENTRATIONS OVER 24 HOURS IN FED AND FASTED HORSES

5.3.1 Objectives

The objective of this study was to investigate the within-day variation in the plasma carnosine and histidine concentrations in resting thoroughbred horses during a normal dietary regimen and following a 12 h overnight fast. A knowledge of the normal within-day variation in plasma carnosine and histidine concentration was also necessary for later pharmacokinetic investigations following oral and intra-venous carnosine administration.

5.3.2 Experimental methodology

Protocol and sampling procedure

Heparinized venous blood samples (5 ml) were collected at 2 hourly intervals via an indwelling catheter from six resting thoroughbred horses, aged 4 - 7 years (5 geldings and 1 filly), over a 24 h period on two separate days. On the first day the horses underwent a normal feeding regimen. Five of the horses were fed a normal diet comprising Spillers Stud Cubes and the sixth was fed Spillers Racehorse Cubes. Feed (approximately 2 kg) was provided three times per day at 07.00, 12.30 and 16.30 h. Hay (2 - 3 kg) was provided with both the morning and evening feeds. Water was provided *ad libitum* throughout the day. On the second day the horses were fasted following the evening feed. The two experimental sessions were separated by a period of two days.

Analytical methods

Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3 (Dunnett and Harris 1992).

Statistical Analysis

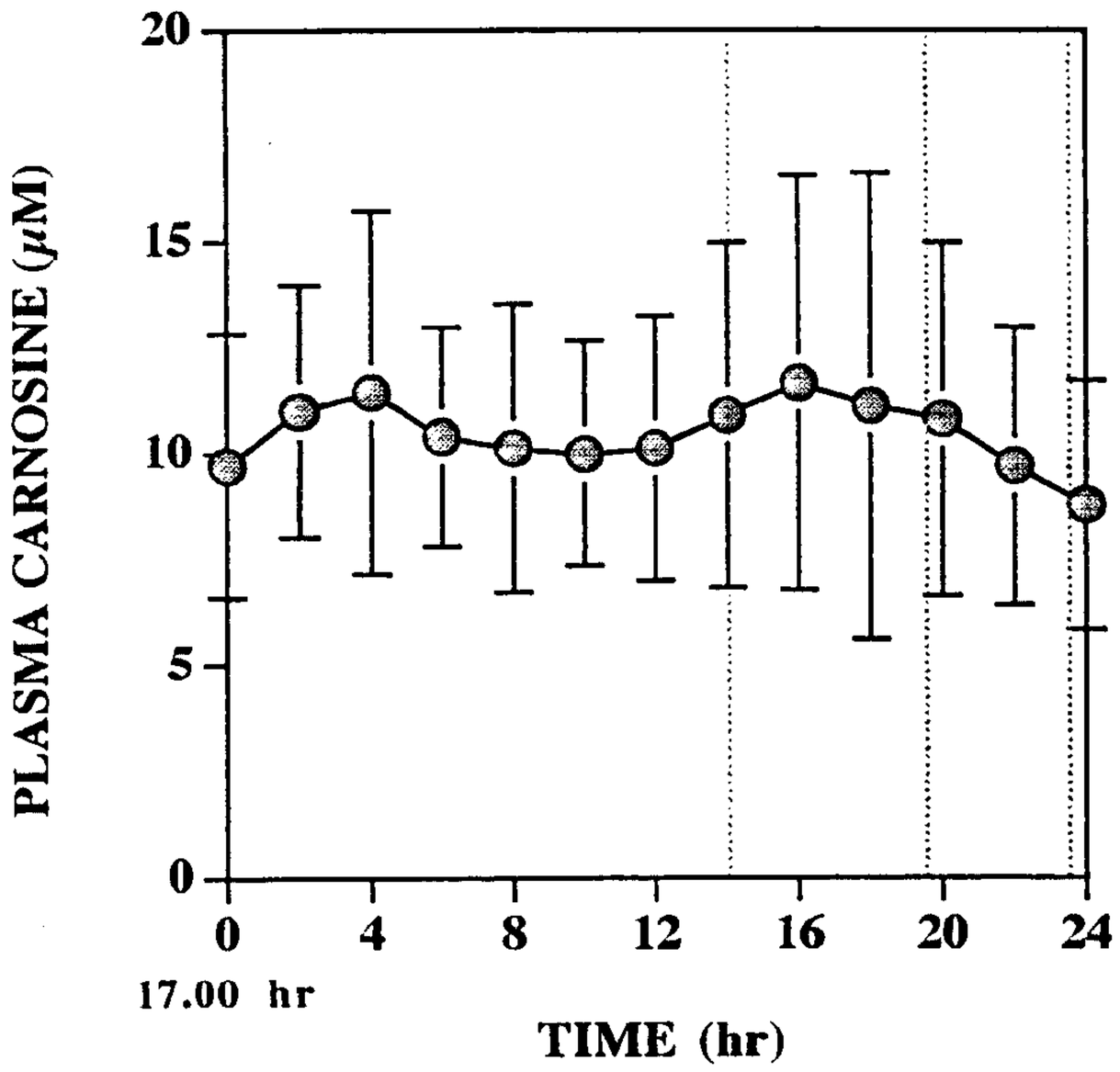
Plasma carnosine and histidine concentrations at different times in fed and fasted horses were compared using Student's *t* -test for unpaired data. Plasma carnosine and histidine concentrations

between fed and fasted horses at different times were compared using Student's *t* -test for paired data.

5.3.3 Results

Large between-horse variations in plasma carnosine and histidine concentrations were evident. However, values for all six horses were within the normal ranges established in the preceding study. The ranges in mean plasma carnosine and histidine concentrations over 24 h in resting horses during the normal feeding regime were 8.8 - 11.7 and 56.9 - 68.5 μM, respectively. No significant changes in mean plasma carnosine or histidine concentrations were found over 24 h in resting horses during normal feeding ($p > 0.05$). The mean within-horse variances in plasma carnosine and histidine concentrations during normal feeding were ± 1.6 and ± 9.0 μM, respectively. Mean (± SD) plasma carnosine and histidine concentrations over 24 h are shown in Figures 5.4 and 5.5, respectively. The ranges in mean plasma carnosine and histidine concentrations over 24 h in resting horses during fasting were 10.0 - 11.7 and 55.8 - 65.6 μM, respectively. No significant changes in mean plasma carnosine and histidine concentrations were found over 24 h in resting horses during fasting ($p > 0.05$). The mean within-horse variance in plasma carnosine and histidine concentrations were ± 1.5 and ± 6.4 μM, respectively. Mean plasma carnosine and histidine concentrations were not significantly different between fed and fasted horses ($p > 0.05$). Comparisons of mean (± SD) plasma carnosine and histidine concentrations between fed and fasted horses over the period 12 - 24 h are shown in Figure 5.6.

Figure 5.4 Variation in mean (± SD) plasma carnosine and histidine concentrations in resting thoroughbred horses over 24 h during a normal feeding regimen (n = 6). (Feeding times are indicated by vertical dotted lines)


PLASMA CARNOSINE (μM)
20
15
10
5
0
0
4
8
12
16
20
24
17.00 hr
TIME (hr)

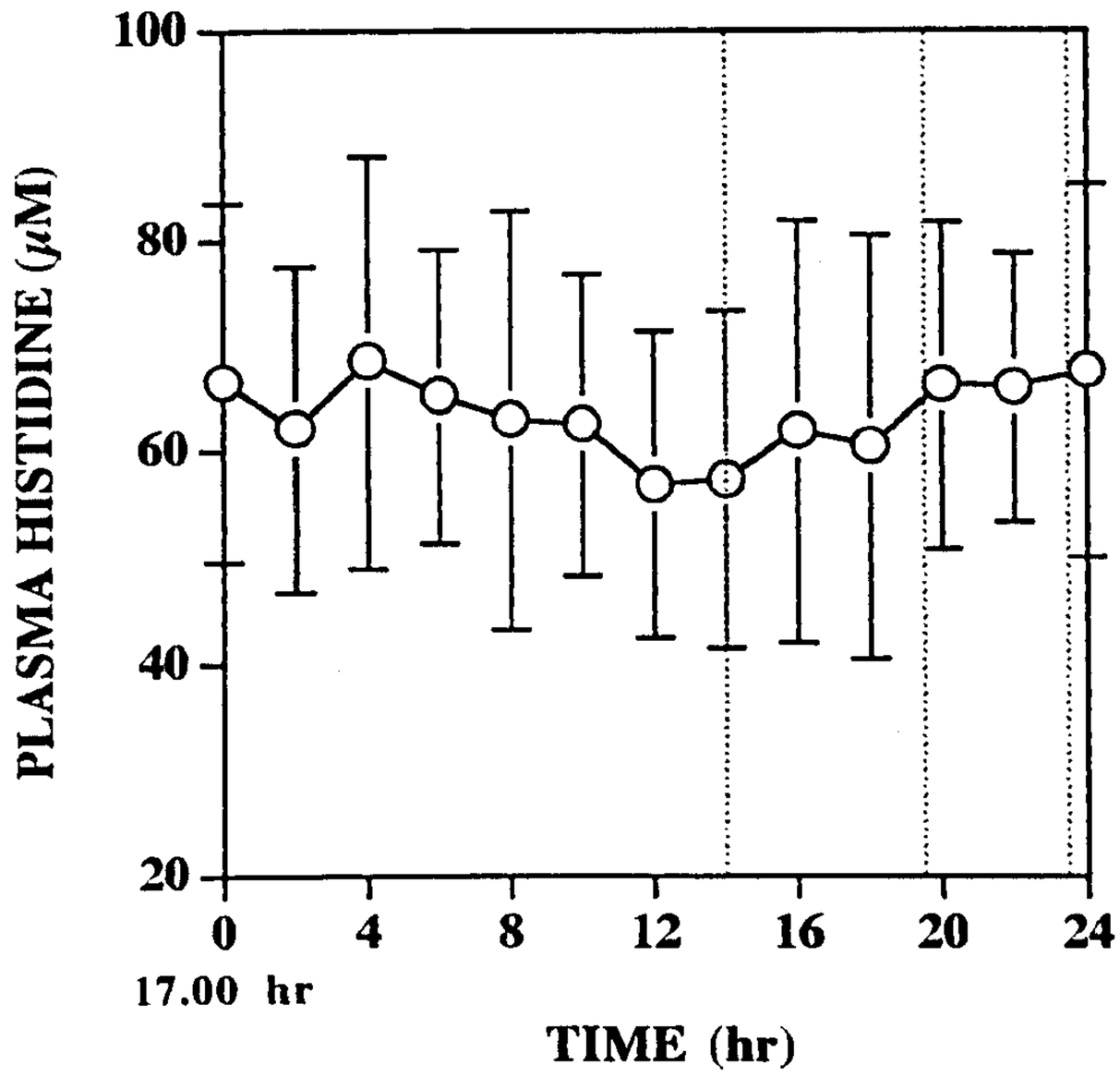
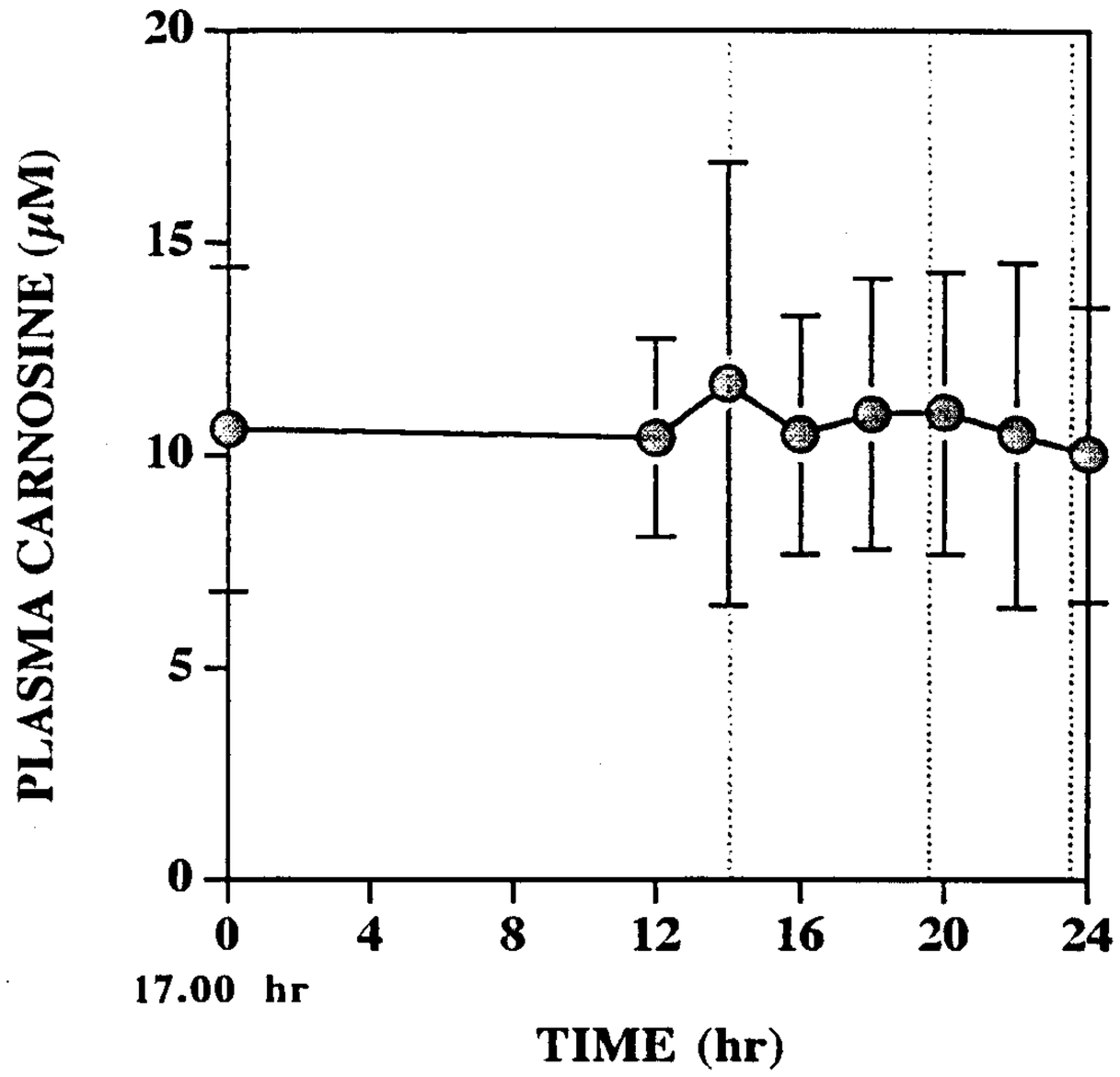

PLASMA HISTIDINE (μM)
100
80
60
40
20
0
4
8
12
16
20
24
17.00 hr
TIME (hr)

Figure 5.5 Variation in mean (± SD) plasma carnosine and histidine concentrations in resting thoroughbred horses during 24 h fasting (n = 6).

(Feeding times are indicated by vertical dotted lines)


PLASMA CARNOSINE (μM)
20
15
10
5
0
0
4
8
12
16
20
24
17.00 hr
TIME (hr)

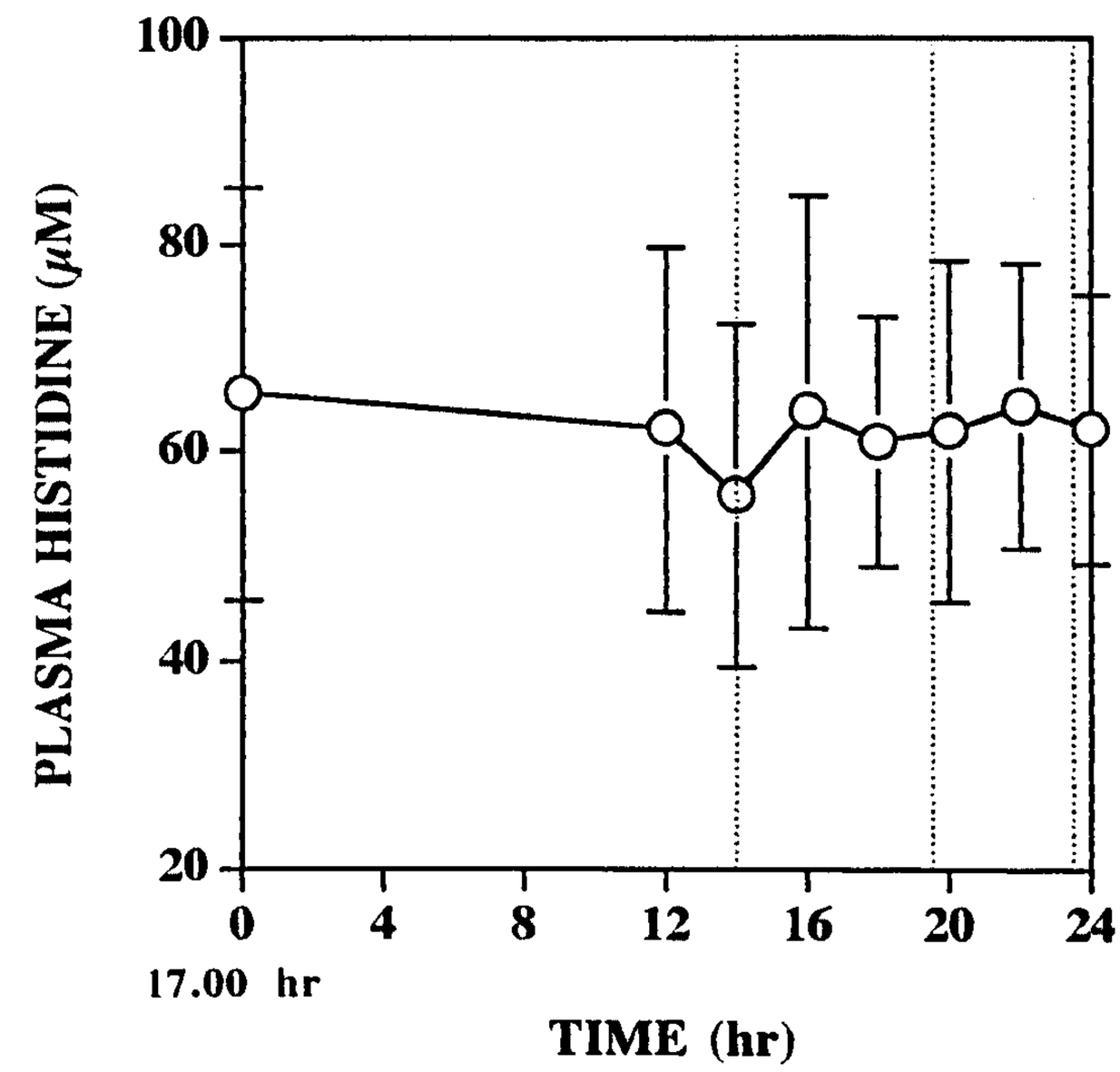
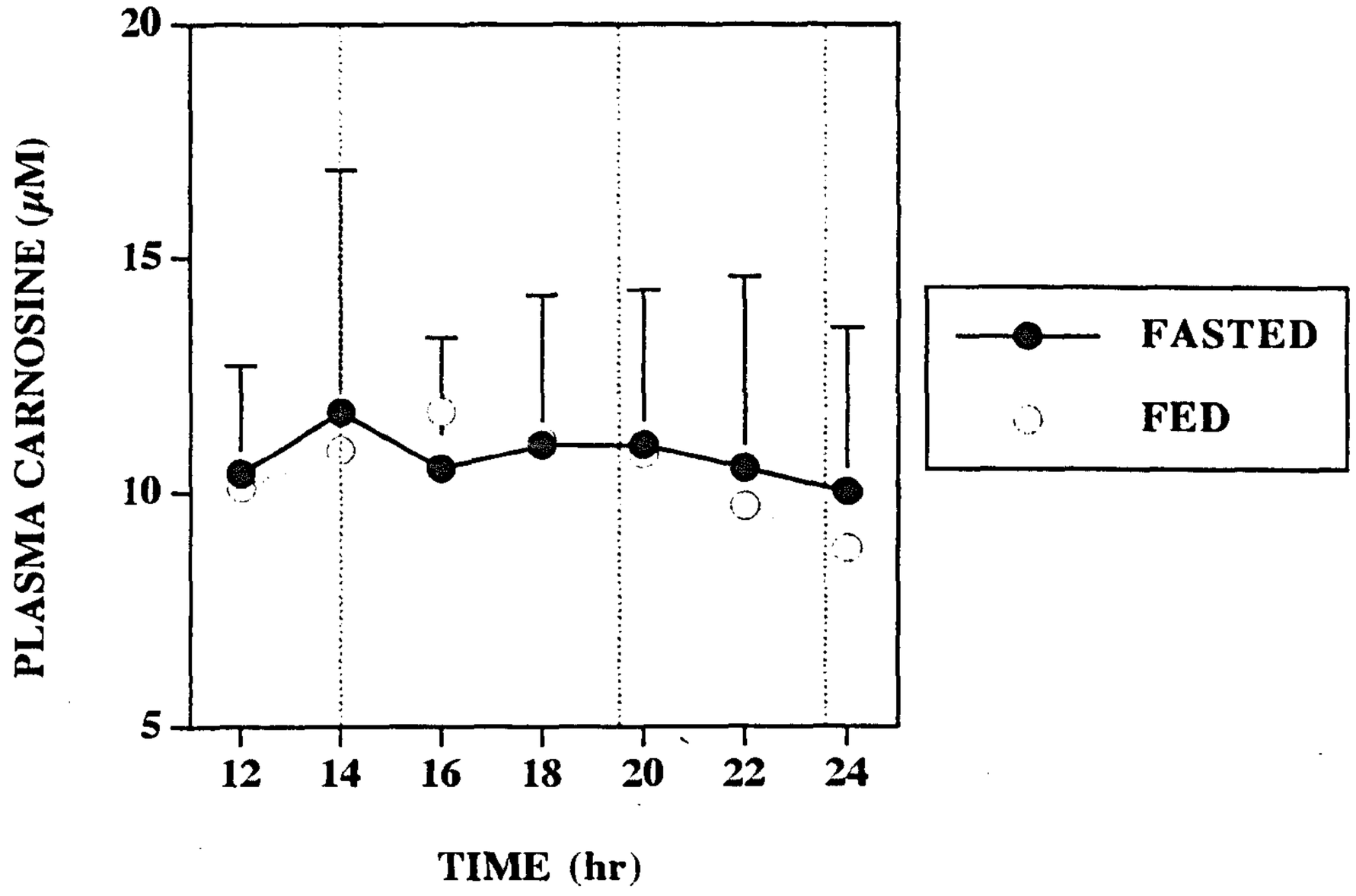
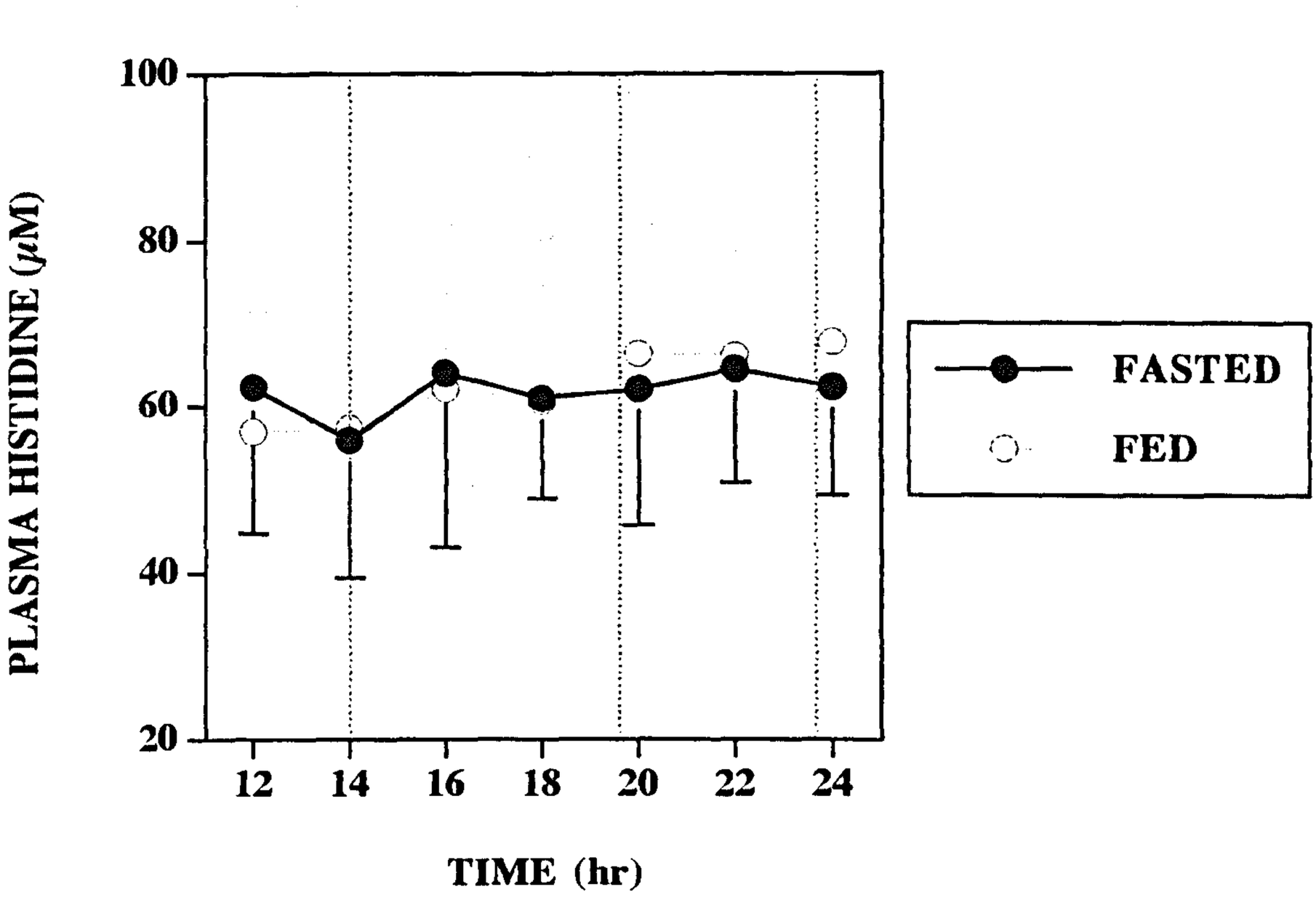

PLASMA HISTIDINE (μM)
100
80
60
40
20
0
4
8
12
16
20
24
17.00 hr
TIME (hr)

Figure 5.6 Comparison of mean (± SD) plasma carnosine and histidine concentrations over 24 h in fed and fasted resting thoroughbred horses (n = 6).

(Feeding times are indicated by vertical dotted lines)


PLASMA CARNOSINE (μM)
20
15
10
5
12
14
16
18
20
22
24
TIME (hr)
FASTED
FED


PLASMA HISTIDINE (μM)
100
80
60
40
20
12
14
16
18
20
22
24
TIME (hr)
FASTED
FED

5.4 STUDY C: VARIATION IN PLASMA CARNOSINE CONCENTRATION AS A RESULT OF HIGH-INTENSITY EXERCISE.

5.4.1 Objectives

The aim of the study was to evaluate whether high-intensity exercise in the thoroughbred horse elicited a change in plasma carnosine concentration.

5.4.2 Experimental methodology

Protocol and sampling procedure

Six thoroughbred horses, NV, NS, OJ, RI, HO and EL (4 geldings, 2 fillies), aged 5 - 13 years, were trained for 4 weeks prior to undertaking a treadmill based standardized exercise test (SET). During the SET the horses were exercised continuously and at an intensity which was increased in a step-wise fashion to a point of near-maximal performance during the exercise protocol. Horses were walked at 1.6 m s^{-1} for 10 min on a level surface followed by a succession of canters of 1 min duration on a 5^{o} incline at speeds of 6, 8, 9, 10, 11 and 12 m s^{-1}, or until the onset of fatigue, as defined by the point at which they were no longer able to match the speed of the treadmill despite humane encouragement. Heparinized venous blood samples (5 ml) were collected immediately prior to exercise and 5 min, 30 min, 2 h and 24 h after exercise.

Analytical methods

Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3. Plasma aspartate transaminase (AST: EC 2.6.1.1) and creatine kinase (CK: EC 2.7.3.2) activities were determined by kinetic methods using commercial diagnostic reagents (AST, Randox Laboratories Ltd., Co. Antrim, N. Ireland.; CK, Kone^{TM} Diagnostics, San Diego, USA.) on a Kone^{TM} Specific Autoanalyser.

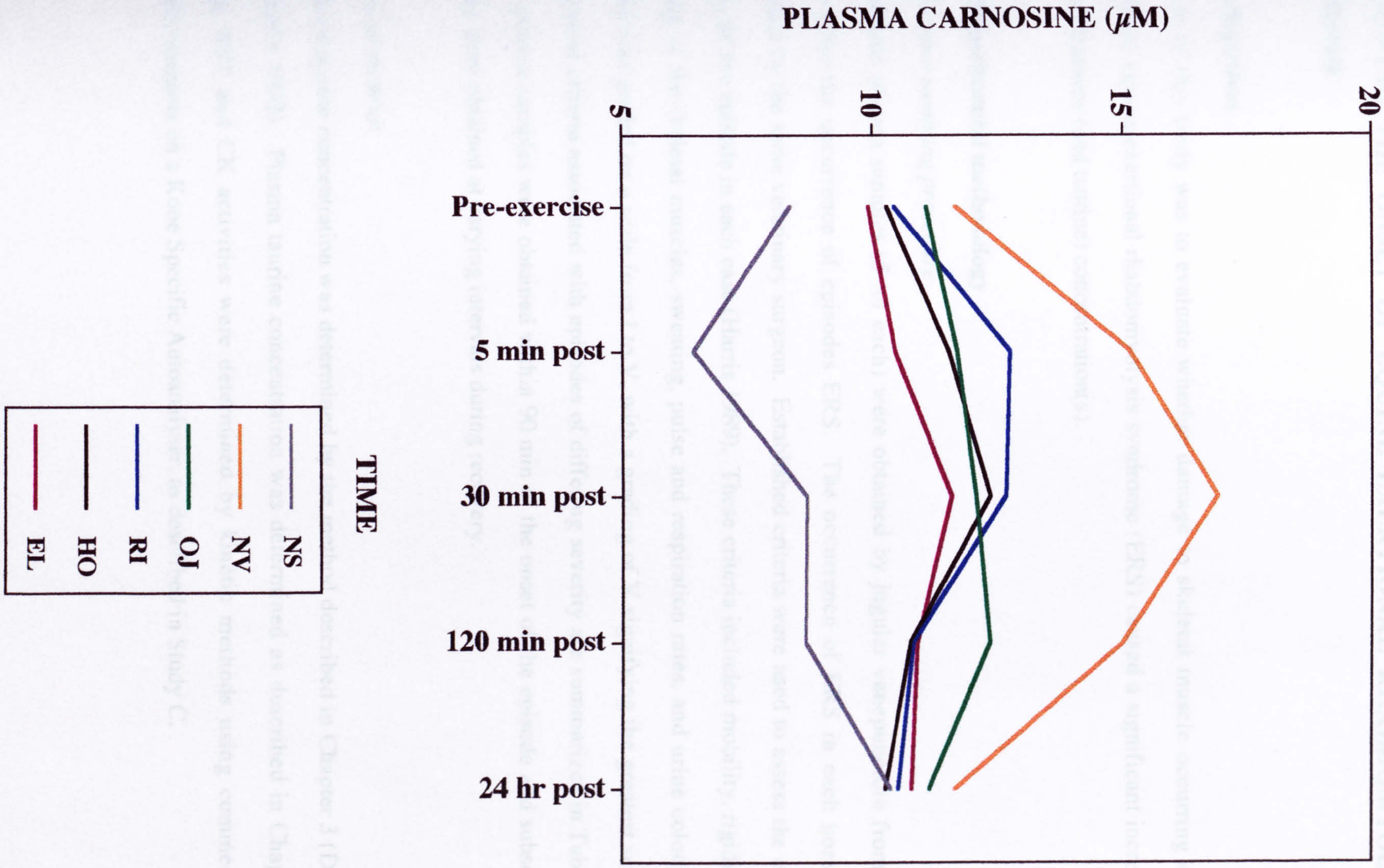
Statistical analysis

Pre- and post-exercise plasma carnosine concentrations were compared using Student's *t* -test for paired data.

5.4.3 Results

The mean (± SD) pre-exercise plasma carnosine concentration (10.3 ± 1.0 μM) was within the normal range established in Study A. Plasma carnosine concentration increased in 5 out of 6 horses following high-intensity exercise. The peak mean plasma carnosine concentration of 12.4 ± 4.4 μM which occurred at 30 min post-exercise was significantly greater than the pre-exercise concentration ($p < 0.05$). However, the significance of this increase was due to the concentration change in one horse (NV). The mean peak concentration only marginally exceeded the upper limit of the normal within-day variation in plasma concentration of thoroughbred horses at rest (8.8 - 11.7 μM), as established in Study B, and was within the normal range for older horses. The greater increase in post-exercise plasma carnosine concentration in horse NV correlated with a greater increase in plasma AST activity in this horse. However, for all horses the mean pre- and post-exercise plasma AST and CK activities were within the normal clinical reference range (AST, 105 - 230 IU l^{-1}; CK, 16 - 49 IU l^{-1}) and were not indicative of the occurrence of skeletal muscle damage. Mean pre- and post-exercise AST and CK activities were not significantly different ($p > 0.05$).

Figure 5.7 Individual plasma carnosine concentrations in thoroughbred horses following high-intensity exercise (n = 6).


PLASMA CARNOSINE (μM)
5
10
15
20
Pre-exercise
5 min post
30 min post
120 min post
24 hr post
TIME
EL
HO
RI
OJ
NV
NS

5.5 STUDY D: CHANGES IN PLASMA CARNOSINE CONCENTRATION FOLLOWING THE ONSET OF EQUINE EXERTIONAL RHABDOMYOLYSIS SYNDROME

5.5.1 Objectives

The aim of this study was to evaluate whether damage to skeletal muscle occurring during episodes of equine exertional rhabdomyolysis syndrome (ERS) caused a significant increase in plasma carnosine (and taurine) concentration(s).

5.5.2 Experimental methodology

Protocol and sampling procedure

Heparinized plasma samples (5 ml each) were obtained by jugular venepuncture from three horses after the occurrence of episodes ERS. The occurrence of ERS in each horse was diagnosed by the same veterinary surgeon. Established criteria were used to assess the clinical severity of the episode in each case (Harris 1989). These criteria included mobility, rigidity and swelling of the skeletal muscles, sweating, pulse and respiration rates, and urine colouration. Severity was graded on a scale from I to V, with a grading of V signifying the greatest severity. The clinical criteria associated with episodes of differing severity are summarized in Table 5.3. Initial plasma samples were obtained within 90 min of the onset of the episode and subsequent samples were obtained at varying intervals during recovery.

Analytical methods

Plasma carnosine concentration was determined by the method described in Chapter 3 (Dunnett and Harris 1992). Plasma taurine concentration was determined as described in Chapter 2. Plasma AST and CK activities were determined by kinetic methods using commercially available reagents on a Kone Specific Autoanalyser, as described in Study C.

Table 5.3 Grading of the severity of ERS episodes and the associated clinical criteria.

Adapted from Harris (1989).

Grade of severity	Clinical criteria					
	Mobility	Muscle condition	Excessive sweating.	Elevated pulse/resp.	Evidence of GIT disturbance.	Discoloured urine.
I	Slight stiffness. Shortened stride.	Appears normal.	-	-	-	-
II	Reluctant to move.	Often appears normal.	±	+	±	±
III	Unable to move.	Firm and swollen. Resent palpation.	+	+	+	+
IV	Unable to move. May become transiently recumbent.	Firm and swollen. May not resent palpation.	++	++	++	++
V	Rapidly become recumbent.	Firm with possible wasting (atrophy) subsequent to the episode.	++	+++	+++	+++

5.5.3 Results

Three cases of ERS were investigated during the course of this study. The clinical details of each case are summarised below.

Case 1 A 17 year-old Thoroughbred X Arab mare (AB) experienced an episode of ERS following approximately 10 - 20 min of moderate exercise comprising walking, trotting and cantering over a series of 6 cross-country fences. The horse showed a reluctance to move, firm and swollen gluteal muscles, and a slight discolouration of the urine. The clinical severity of the episode in this horse was graded II - III.

Case 2 An 11 year-old Thoroughbred mare (EL) experienced an episode of ERS following moderate exercise comprising approximately 40 min walking, 15 min trotting and 5 min half-speed up-hill cantering. The horse lay down immediately on returning to the stables after exercise but could be encouraged to stand and move. The clinical severity of the episode in this horse was graded II.

Case 3 A 10 year-old Thoroughbred X gelding (PD). Details of the exercise undertaken immediately prior to the occurrence of the ERS episode are not known. Some slight stiffness was evident but no muscle abnormality was apparent. All other clinical signs appeared normal. The clinical severity of the episode in this horse was graded I.

The peak increases in plasma carnosine and taurine concentrations, and AST and CK activities following the onset of ERS in each of the three cases are detailed in Table 5.4. Changes in plasma carnosine and taurine concentrations, and AST and CK activities with respect to time following the episodes of ERS in the individual horses are shown in Figure 5.8. Peak plasma carnosine concentrations were significantly elevated (5 - 70-fold) above the normal range in all three horses. The increases in plasma carnosine and taurine concentrations, and AST and CK activities varied considerably between the individual horses and did not necessarily correlate with the severity of the clinical signs in each case. A large increase in plasma taurine

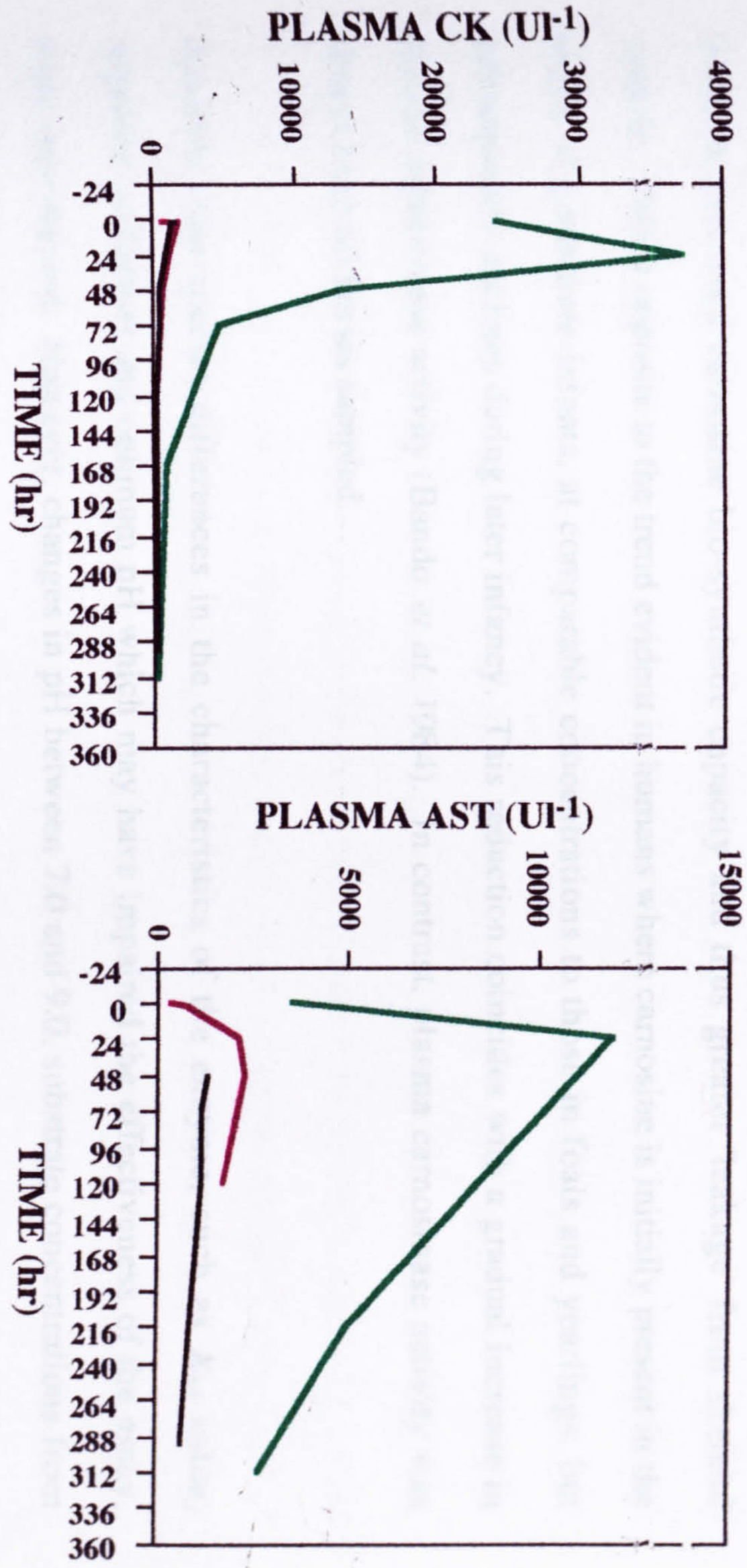
concentration was evident in only one of the horses (PD). The significance of the much smaller increases in the other two horses was difficult to assess in the absence of sufficient data relating to the normal range and possible within-day variation in equine plasma taurine concentration. Plasma AST and CK activities, which are often used as indices of muscle damage, were significantly increased above the normal range (see previous study). The highest values for all parameters were evident in horse PD. Although only three horses were studied when the data were combined there were close correlations between peak plasma carnosine concentration and both peak plasma AST and CK activities. Similar correlations were found between peak plasma taurine concentration and both peak plasma AST and CK activities. The peak plasma carnosine concentration occurred approximately 1 - 2 h after the onset of ERS as did peak plasma CK activity in the two horses with the lower increases in CK (AB and EL). In contrast, peak plasma AST activities occurred approximately 24 - 48 h after the ERS episode. There was a rapid initial clearance of carnosine from the plasma, although concentrations did not return to values close to the normal range for several days.

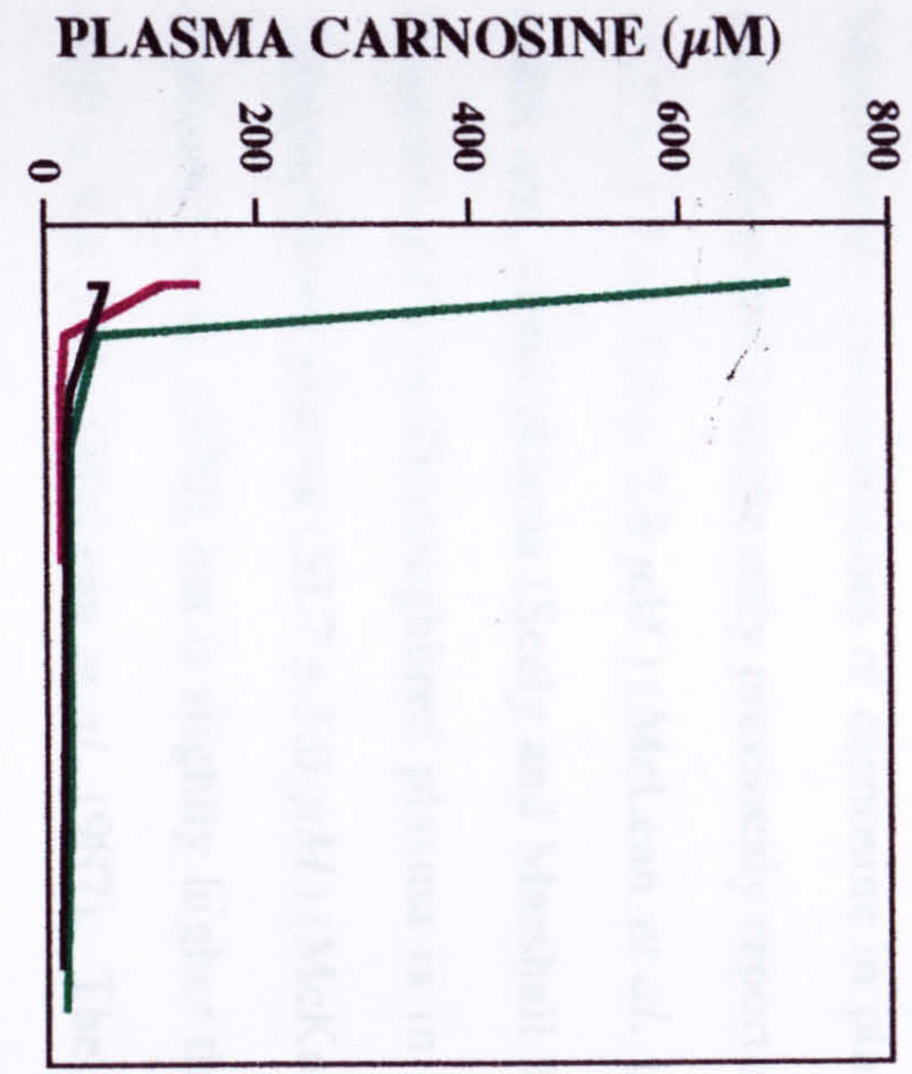
Table 5.4 Peak values in plasma carnosine and taurine concentrations, and AST and CK activities following the onset of ERS in three cases.

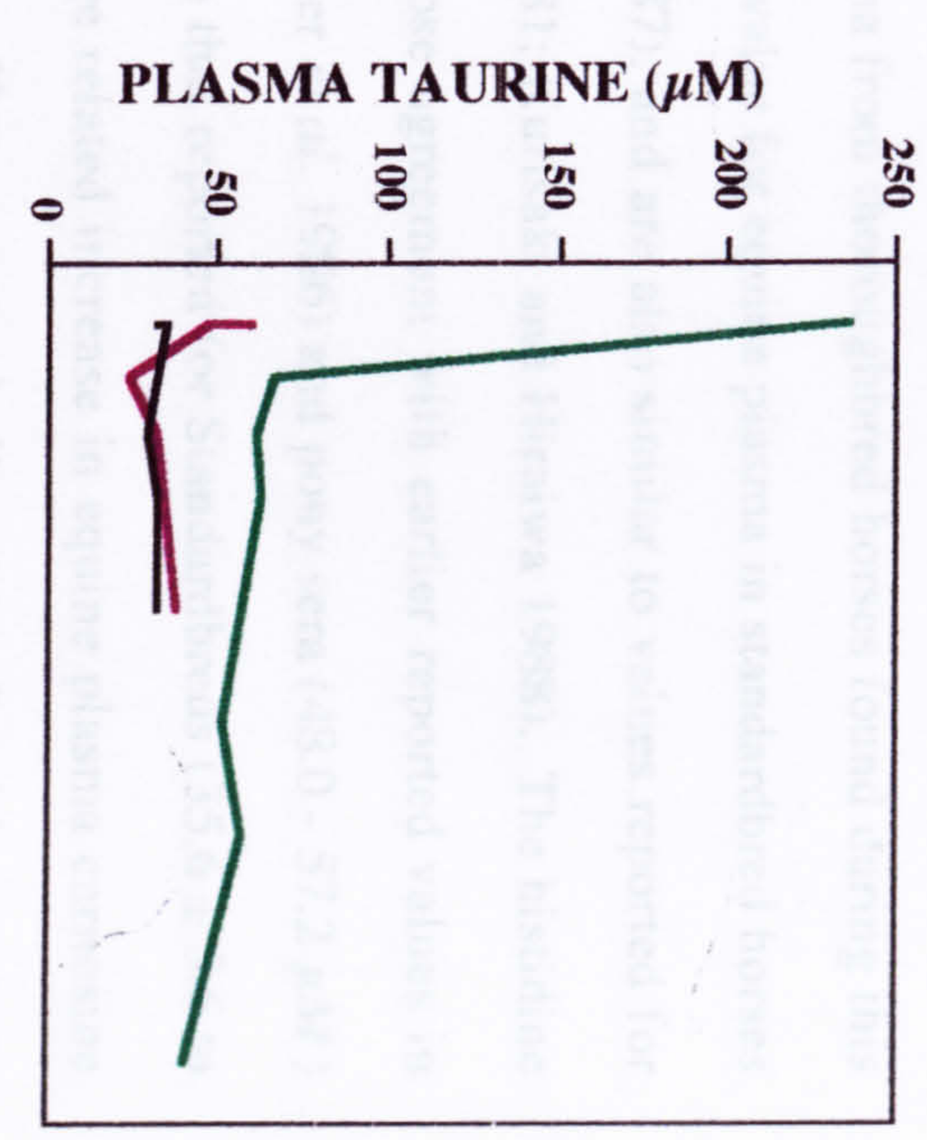
Case	Grade	Severity	Peak values measured (time after onset)			
			Carnosine, μM	Taurine, μM	CK, IU l^{-1}	AST, IU l^{-1}
AB	II-III	Moderate	57.6 (2 h)	38.4 (12 d)	1500 (1 h)	1250 (48 h)
EL	II	Mild	143.0 (1 h)	63.1 (2 h)	1700 (2 h)	2300 (48 h)
PD	I	Mild	702.3 (1 h)	256.0 (1 h)	37000 (24 h)	11900 (24 h)

Figure 5.8 Changes in plasma carnosine and taurine concentrations, and AST and CK activities with respect to time following the onset of ERS in the individual horses.


AB
EL
PD


PLASMA CK (UI-1)
0
10000
20000
30000
40000
TIME (hr)
-24
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
PLASMA AST (UI-1)
0
5000
10000
15000
TIME (hr)
-24
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360


PLASMA CARNOSINE (μM)
0
200
400
600
800


PLASMA TAURINE (μM)
0
50
100
150
200
250

5.6 DISCUSSION

Measurements of plasma carnosine concentrations have been performed in very few species. Micromolar concentrations of carnosine in plasma from thoroughbred horses found during this study are similar to the only previously reported value for equine plasma in standardbred horses (1.7 ± 1.7 to 5.2 ± 2.8 μM) (McLean *et al.* 1987), and are also similar to values reported for chick and rabbit plasma (Seely and Marshall 1981; Kurisaki and Hiraiwa 1988). The histidine concentration in Thoroughbred plasma is in close agreement with earlier reported values in Thoroughbred plasma (53.7 ± 3.0 μM) (McKeever *et al.* 1986) and pony sera ($48.0 - 57.2$ μM) (Reitnour *et al.* 1970), but is slightly higher than that reported for Standardbreds (35.6 ± 5.6 to 40.9 ± 9.4 μM) (McLean *et al.* 1987). The age related increase in equine plasma carnosine concentration has not previously been reported. However, a similar age related increase in equine plasma carnitine concentration has been reported (Foster *et al.* 1989). Carnosine has been reported to be synthesized within the skeletal muscle of some vertebrates. Assuming a similar situation exists in the horse, the age-related increase plasma carnosine concentration may arise from an increased carnosine bio-synthetic capacity and thus greater 'leakage' from skeletal muscle. This is opposite to the trend evident in humans where carnosine is initially present in the blood of premature infants, at comparable concentrations to those in foals and yearlings, but subsequently declines during later infancy. This reduction coincides with a gradual increase in plasma carnosinase activity (Bando *et al.* 1984). In contrast, plasma carnosinase activity was absent from all horses sampled.

Possible inter-species differences in the characteristics of the enzyme, such as K_M value, requisite co-factors and optimum pH which may have impaired the effectiveness of the assay, were investigated. However, changes in pH between 7.0 and 9.0, substrate concentrations from $0.5 - 50$ mM , and the presence or absence of co-factors and stabilizers, including Mn^{2+}, Co^{2+} and Cd^{2+} (2 mM), and DTT (1 mM), failed to indicate the existence of plasma carnosinase activity. The absence of plasma carnosinase activity in the thoroughbred horse is consistent with earlier data from other non-primates including equine serum from an undisclosed breed (Jackson *et al.*

1991). The presence of carnosine in equine plasma is consistent with the absence of plasma carnosinase activity.

In both humans and horses it has been established that the plasma concentrations of certain metabolites, such as glucose, triiodothyronine, free fatty acids, cortisol and growth hormone exhibit regular fluctuations during the course of the day (Gibson *et al.* 1985; Orme *et al.* 1994; Youket *et al.* 1985; Zilva and Parnall 1984). Such changes can be related to feeding. Increases in plasma concentrations of proteinaceous amino acids occur as a result of normal food intake, as has been demonstrated in lambs and poultry (Featherston 1972; Nimrick *et al.* 1971), and consumption of diets containing an excess of a given amino acid cause produce an increase in the plasma concentration of that amino acid (Zimmerman and Scott 1975). The higher mean within-horse variance in plasma histidine concentration in normally fed horses (9.0 μM) compared with fasted horses (6.4 μM) suggests that normal feeding does to some extent induce alterations in circulating concentrations of histidine. This is consistent with data from previous studies in horses which showed post-feeding increases in the plasma concentrations of amino acids in general (Johnson and Hart 1974; Russell *et al.* 1986) and histidine in particular (Johnson and Hart 1974). Furthermore, Johnson and Hart (1974) reported a significant reduction in plasma histidine concentration fasting horses. However, no reduction in mean plasma histidine concentration during fasting was observed in the present investigation. This apparent discrpency may be due to the use of resting horses in the present study, unlike the fomer, in which the horses exercised for 2 - 4 h at low to moderate intensity during the experimental period. Comparison of the within-horse variance in plasma carnosine concentration between fed and fasted horses (1.6 μM fed, 1.5 μM fasted) suggests that normal feeding has very little if any influence on circulating concentrations of carnosine.

In conclusion, there appeared to be little influence of feeding on plasma carnosine concentrations. The within-day fluctuations in plasma carnosine concentration were random and differences in concentration at different times were not statistically significant ($p > 0.05$). The within-day variation in plasma histidine concentration was random and differences in

concentration at different times were not statistically significant ($p > 0.05$). However, normal feeding appeared to increase the within-day variation in plasma histidine concentration.

Post-exercise increases in carnosine concentrations in equine plasma were reported briefly in a previous investigation of changes in plasma amino acid concentrations in two groups of five standardbred racehorses before and after racing. In one group where post-exercise samples were collected between 2 and 5 min after racing, the pre- and post-exercise plasma carnosine concentrations were 5.2 ± 2.8 and 11.0 ± 9.1 μM, respectively. In the second group where post-exercise samples were collected between 2 and 3 h after racing, the pre- and post-exercise concentrations were 1.7 ± 2.7 and 8.3 ± 3.3 μM, respectively (McLean *et al.* 1987).

It is possible that increases in plasma carnosine concentrations after high-intensity exercise may arise from muscle cell membrane damage and the subsequent transfer of the cellular contents into the circulation. Owing to the presence of very high concentrations of carnosine within equine skeletal muscle significant increases in plasma carnosine concentrations may arise as a consequence of even minor muscle fibre damage. Although no previous investigations appear to have addressed this point in association with exercise, 10 to 20-fold increases in plasma carnosine and anserine concentrations have been reported following traumatic shock induced skeletal muscle damage in rats (Kurisaki and Hiraiwa 1988). Study C indicates that high-intensity exercise *per se* does not result in large increases in plasma carnosine concentration. Evidence from this study however, suggests that large increases in plasma carnosine concentration may occur as a result of skeletal muscle damage associated with ERS, and that the peak concentration is probably proportional to the severity or extent of the damage. The use of pharmacokinetic parameters determined after intra-venous injection of carnosine could enable an estimate to be made of the mass of muscle damaged. However, it would not be possible to discriminate between mild but widespread damage, and severe localized damage.

Owing to the almost exclusive localization of taurine in type I muscle fibres and the much greater concentration of carnosine in type II fibres, the appearance of significantly elevated plasma

carnosine or taurine concentrations (assuming other causes of plasma taurine elevation are eliminated) may be indicative of selective type I or type II fibre damage, respectively. In the one horse (PD) large increases in both plasma AST and CK activities are indicative of extensive skeletal muscle damage. The large increases also seen in plasma carnosine and taurine concentrations suggest the involvement of both type I and type II fibre damage. This contradicts the earlier suggestion that muscle damage associated with episodes of ERS is confined to the type II muscle fibres (Valberg *et al.* 1993). However, considerable previous investigations have attested to the ubiquitous nature of taurine in mammalian tissues in many species (Jacobson and Smith 1968) and although no such studies appear to have been conducted in the horse, it is reasonable to assume that a similar widespread distribution of taurine in equine tissues exists. In view of this it may be difficult to attribute increases in plasma taurine concentrations solely to skeletal muscle damage.

Plasma carnosine concentrations may offer a more reliable estimation of the degree of muscle damage incurred than plasma AST and CK activities. Neither plasma AST or CK activities are indicative of damage specifically to the skeletal muscles. Although CK activities are relatively high in skeletal muscle, significant activities are also present in the brain and heart. AST activity is not specific to skeletal muscle but also occurs at significant levels within the heart and liver. Hence, increases in these parameters are also likely with various forms of soft tissue damage. Owing to the relatively low concentration of carnosine in tissues other than skeletal muscle, damage to these other tissues is unlikely to result in increases in plasma carnosine concentrations which could be misinterpreted as skeletal muscle damage. For example, from the data given in Chapter 4, it can be estimated that complete destruction of the myocardium would result in a peak plasma carnosine concentration of only 30 - 40 μM .

CHAPTER 6

METABOLISM OF CARNOSINE AND N-α-ACETYLCARNOSINE FOLLOWING ORAL AND INTRA-VENOUS ADMINISTRATION IN THE THOROUGHBRED HORSE

6.1 INTRODUCTION

Carnosine metabolism has been investigated using both *in vitro* and *in vivo* techniques. *In vivo* metabolic studies, predominantly in rats and humans, but also in trout, have involved the administration of carnosine by intra-muscular injection or orally via naso-gastric intubation or as a supplement to the normal feed. There appears to be no previous studies describing the metabolism of carnosine *in vivo* following administration via the intra-venous route. *In vitro* studies, focusing mostly on carnosine transport, have been conducted utilizing isolated kidney and intestinal tissue from several species including rats, mice, guinea pigs and rabbits. The transport, metabolism and excretion of carnosine and the other imidazole dipeptides varies markedly between species.

Carnosine and other imidazole dipeptides are absent from fasting human plasma and are present at only very low concentrations in the urine of normal subjects. They are however, detectable following ingestion of meals with a high meat content, although plasma concentrations are still low. Perry *et al.* (1967) reported plasma carnosine concentrations of between 6 and 18 μM, in 2 normal subjects, 2 h after ingestion of between 16 and 20 g d^{-1} kg^{-1} BW of chicken, a rich dietary source of carnosine. Approximately 3 - 4 mmol d^{-1} of carnosine was also excreted in the urine, in addition to smaller quantities of the primary metabolites histidine and ß-alanine (Perry *et al.* 1967). Similar results describing the urinary excretion of balenine and anserine have also been reported following the ingestion of various other meat sources of these imidazole dipeptides, including whale, tuna and pork (Undrum *et al.* 1982; Abe *et al.* 1993). Oral administration of the carnosine in normal human subjects resulted in large increases in plasma histidine and ß-alanine concentrations (Asatoor *et al.* 1970; Sadikali *et al.* 1975; Cook 1976; Gardner *et al.* 1991). No plasma carnosine was detected however, unless cooling procedures were employed during and following sample collection to inhibit plasma carnosinase activity (Gardner *et al.* 1991). Urinary carnosine excretion was rapid and highly variable with 2 - 14% of the administered dose appearing as carnosine and up to a further 2% appearing as ß-alanine in the urine within 5 h of administration (Gardner *et al.* 1991). Both carnosine and anserine are absorbed intact from the GIT in rats following force feeding of large doses, up to 4500 mg kg^{-1} BW (Hama *et al.* 1976),

although at lower doses of approximately 3500 and 350 mg kg^{-1} BW partial hydrolysis of carnosine to its constituent amino acids occurred within the small intestine of the rat with greater hydrolysis being found at the lower dose (Tamaki *et al.* 1985).

In vitro studies have shown that active trans-membrane carnosine transport mechanisms exist in the GIT of several species including the hamster (Matthews *et al.* 1974), rat (Nutzenadel and Scriver 1976), rabbit (Ganapathy and Leibach 1983), mouse (Rajendran *et al.* 1984) and guinea pig (Himuki 1985). Active carnosine transport has also been demonstrated in kidney of rabbits (Ganapathy and Leibach 1982; Ganapathy and Leibach 1983) and rats (Nutzenadel and Scriver 1976). Large doses of carnosine or anserine (approximately 1500 mg kg^{-1} BW) administered by injection into trout white muscle were rapidly removed from this tissue and transferred predominantly to the blood and kidney, with lesser amounts appearing in red muscle and liver. Following carnosine and anserine injection increases in the histidine and 1-methylhistidine concentrations, respectively, were observed in all tissues (Abe 1991).

No previous studies to investigate carnosine transport or metabolism have been conducted in the horse either *in vivo* or *in vitro*. Furthermore, data on the bioavailability of carnosine from the GIT and its metabolic fate are necessary in order to predict the effectiveness of long-term dietary carnosine supplementation as a method to enhance the endogenous skeletal muscle concentration.

6.2 STUDY A: DETERMINATION OF CARNOSINE PHARMACOKINETIC PARAMETERS FOLLOWING INTRA-VENOUS ADMINISTRATION IN THE THOROUGHBRED HORSE.

6.2.1 Objectives

The aims of this study were to measure changes in plasma and urine carnosine concentrations following bolus injection of carnosine in the thoroughbred horse, and subsequently to determine the pharmacokinetic parameters of carnosine clearance from the plasma and to examine aspects

of carnosine metabolism *in vivo* by measuring changes in the plasma and urine concentrations of its metabolites, histidine and ß-alanine.

6.2.2 Experimental methodology

Protocol and sampling procedures

Six experimental thoroughbred horses (5 geldings, 1 filly) aged from 4 - 9 years were used. All horses were fasted overnight, for a minimum period of 12 h, prior to undertaking the study and received no feed during the course of the experiment. Water was, however, provided *ad libitum* . Furthermore, the horses undertook no exercise on the day of the study. Horses were weighed on the morning of the study to enable doses of carnosine to be administered on a body weight basis. Carnosine dissolved in physiological saline (50 ml) was sterilized and administered by intra-venous bolus injection through a Millex^{TM} GS 0.22 μm sterile Teflon^{TM} filter (Millipore UK Ltd., Watford, UK) and 16 gauge catheter inserted into the right jugular vein. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 hr prior to administration. Carnosine was administered at a dose of 20 mg kg^{-1} BW (approximately 10 g total dose). Pre-administration blood samples were collected immediately prior to the carnosine injection, and subsequent blood samples were collected at 5, 10, 20, 40, 60, 120, 240, 360 and 480 min. Urine samples were collected from geldings only over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988).

Analytical methods

Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine ß-alanine concentrations were determined as described in Chapter 2.

Pharmacokinetic and statistical analysis

The disposition kinetics of drugs and other substances administered to animals and humans are in general adequately described by a two compartment model, in which the administered dose

initially enters the central compartment comprising the blood and the extra-cellular fluids of high blood-flow tissues, such as the heart, lungs, liver and kidneys. From here distribution to the peripheral compartment occurs, which comprises low blood-flow tissues, such as the muscles and the skin. Elimination subsequently occurs from the central compartment. Drug movement between these compartments generally conforms to first-order kinetics, hence the rate of drug removal from a given compartment is proportional to the drug concentration within that compartment, and the concentration decreases exponentially with time. Following bolus intravenous injection the change in plasma concentration (C_p) with time (t) can be described mathematically by the general biexponetial equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B are the zero time concentrations for the component distribution and elimination phases, and α and ß are the overall distribution and elimination rate constants, respectively. The empirical values for these four constants (A, B, α, ß) can be obtained by curve stripping of a semi-logarithmic plot (ln C_p vs. t) from the experimental data. Estimates of the following common pharmacokinetic parameters can then be calculated from the empirical constants:

$C_0 = A + B$	(Plasma concentration at t = 0)
$V_c = $ Dose/A+B	(Volume of the central compartment)
AUC = A/a + b/ß	(Area under C_p vs. t curve)
$k_{21} = (A\alpha + B\beta)/(A+B)$	(Micro-rate constant of distribution from peripheral to central compartment)
$k_{10} = \alpha\beta/k_{21}$	(Micro-rate constant of elimination)
$k_{12} = a + \beta - k_{21} - k_{10}$	(Micro-rate constant of distribution from central to peripheral compartment)
$V_{d(ss)} = V_c (1 + k_{12}/k_{21})$	(Volume of distribution at steady state)
$V_{d(area)} = $ Dose/AUC . ß	(Volume of distribution)
$CL_B = k_{10} . V_c$	(Total body clearance)
$MRT = V_{d(ss)}/CL_B$	(Mean residence time)

$AUMC = MRT \,.\, AUC$ (Area under the first moment curve of $C_p \,.\, t$ vs. t)

$t_{1/2(\alpha)} = \ln 2/\alpha$ (Distribution half-life)

$t_{1/2(\beta)} = \ln 2/\beta$ (Elimination half-life)

Pre-administration plasma carnosine and histidine concentrations were subtracted from post-administration concentrations prior to the determination of the pharmacokinetic parameters. Mean (± SD) pharmacokinetic parameters were calculated from parameters estimated in individual horses.

6.2.3 Results

The validity of a two compartment model to describe the change in plasma carnosine concentration with respect to time, in the thoroughbred horse following administration by bolus intra-venous injection, was tested on the mean data for all horses. A plot of $\ln C_p$ vs. t produced a straight line for the terminal (elimination phase), i.e. C_p decreases exponentially with time. Interpolation of this line to $t = 0$ allowed estimates of B (y-axis intercept) and ß (gradient) to be made. Stripping of this line from the original $\ln C_p$ vs. t curve produced a straight line representing the initial (distribution phase). Interpolation of this line to $t = 0$ allowed estimates of A (y-axis intercept) and α (gradient) to be made, as shown in Figure 6.1. Superposition of these two linear components resulted in a theoretical model curve which was a close approximation of the actual plasma clearance curve (Figure 6.2).

The mean plasma carnosine concentration vs. time curve following bolus intra-venous carnosine injection in the thoroughbred horse was described mathematically by the biexponential equation:

$$C_p = 629.5e^{-0.0251t} + 131.1e^{-0.0050t}$$

The mean (± SD) measured plasma carnosine concentration 5 min after intra-venous injection ($671.8 \pm 80.1\ \mu M$) was very close to the value estimated from the pharmacokinetic data (683.1 μM). The mean (± SD) pharmacokinetic parameters are given in Table 6.1. There was only a

small variation in the plasma carnosine clearance curves between individual horses, as shown in Figure 6.3.

Changes in plasma histidine concentrations with time in the individual horses following intra-venous carnosine administration are shown Figure 6.4. There was a large between-horse variation in plasma histidine concentration. There was no detectable ß-alanine found in the plasma of any horse following intra-venous carnosine administration.

Large concentrations of carnosine, up to 17 m*M*, were detected in urine from individual thoroughbred horses following intra-venous carnosine injection. Urine samples collected from 4 of the horses (all geldings) over the 12 hr period indicated that up to 36% of the administered carnosine dose was excreted unmetabolized in the urine, although the percentage recovered was highly variable between horses, as shown in Figure 6.5. Only low concentrations of histidine were detected in urine from the individual horses following intra-venous carnosine injection, as shown in Figure 6.9.

Figure 6.1 Interpolated distribution and elimination components of the ln plasma carnosine concentration vs. time curve in the thoroughbred horse following intra-venous bolus injection at dose of 20 mg kg^{-1} BW.

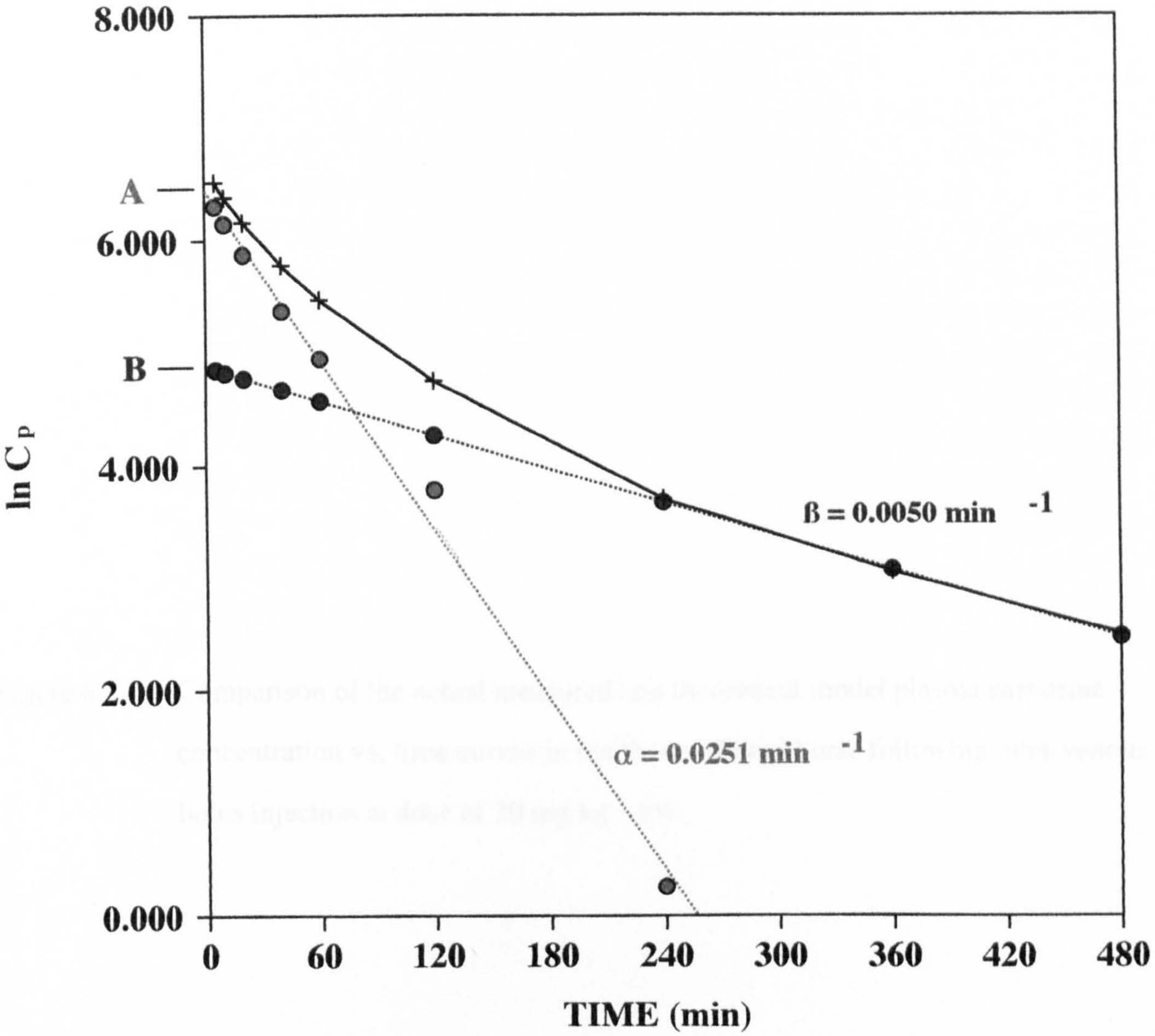

8.000
A
6.000
B
4.000
2.000
0.000
ln Cp
0
60
120
180
240
300
360
420
480
TIME (min)
β = 0.0050 min -1
α = 0.0251 min -1
MEASURED CONCENTRATION
ELIMINATION COMPONENT
ln Cp = -0.0050t + 4.8762
DISTRIBUTION COMPONENT
ln Cp = -0.0251t + 6.4449

Figure 6.2 Comparison of the actual measured and theoretical model plasma carnosine concentration vs. time curves in the thoroughbred horse following intra-venous bolus injection at dose of 20 mg kg^{-1} BW.

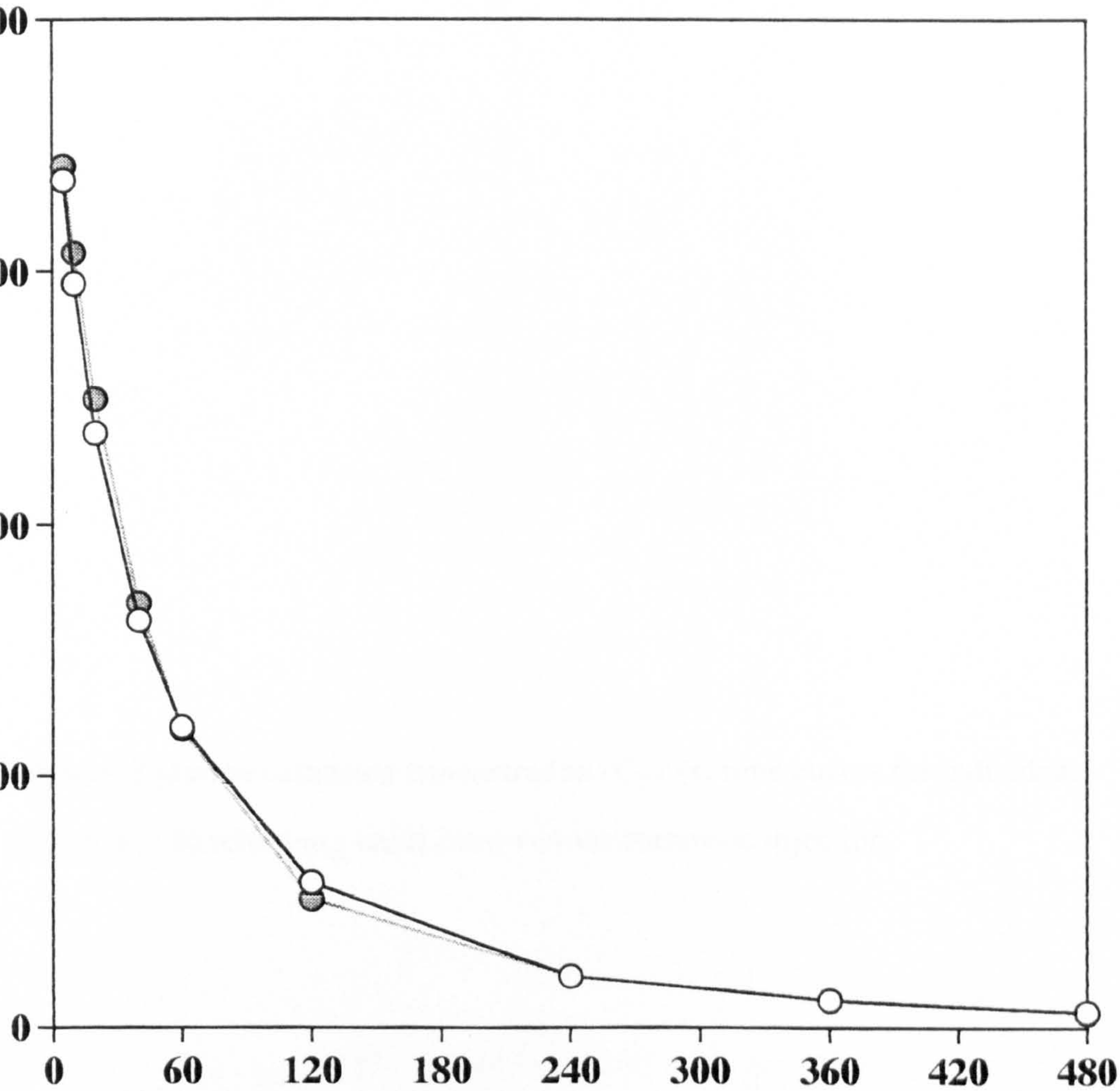

PLASMA CARNOSINE, C p (μM)
800
600
400
200
0
0
60
120
180
240
300
360
420
480
TIME (min)
MEASURED CONCENTRATION
PREDICTED CONCENTRATION
Cp = 629.5e-0.0251t + 131.1e-0.0050t

Figure 6.3 Measured plasma carnosine concentration (C_p) vs. time curves for individual horses (n = 6) following bolus intra-venous carnosine injection.

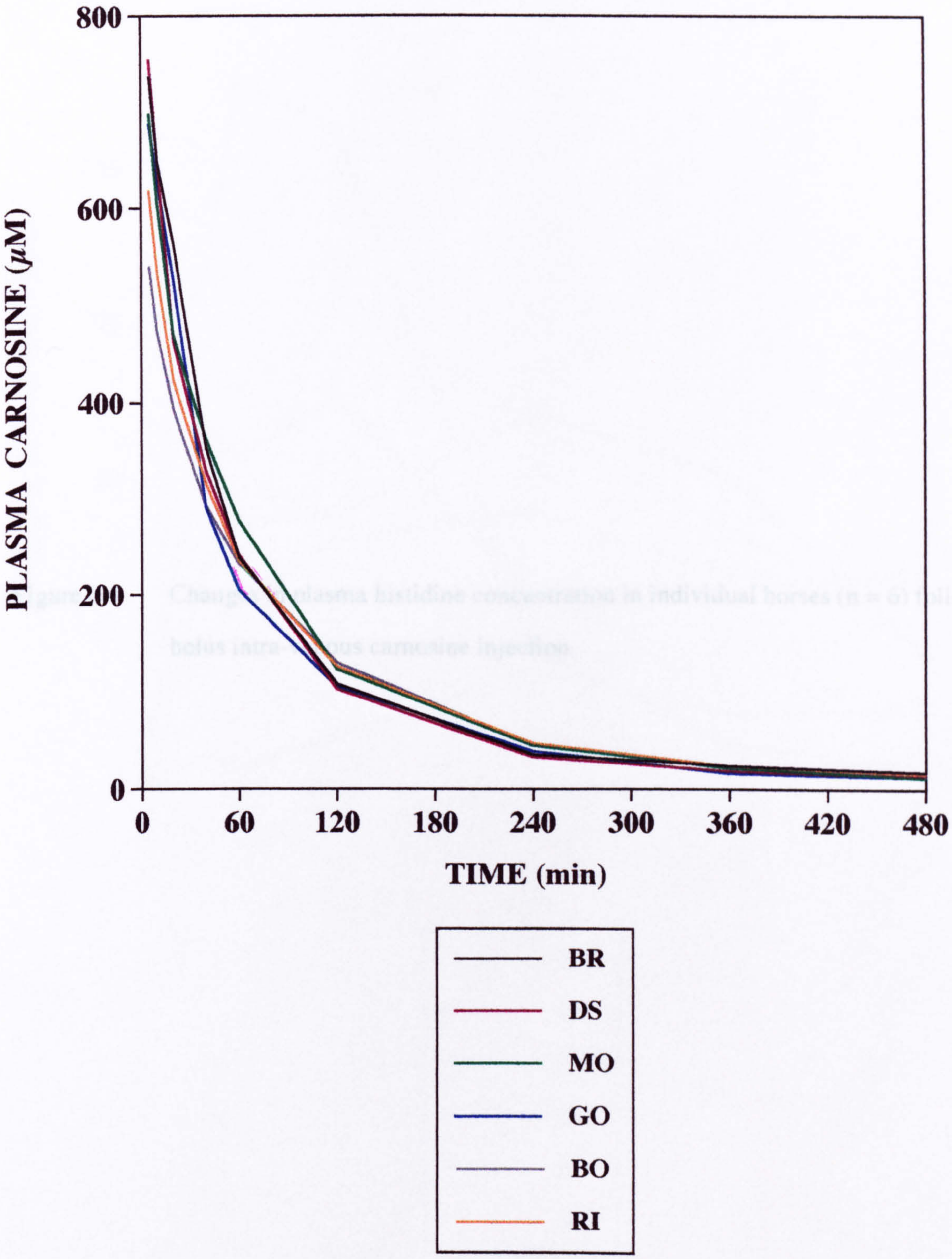

800
600
400
200
0
PLASMA CARNOSINE (μM)
0
60
120
180
240
300
360
420
480
TIME (min)
BR
DS
MO
GO
BO
RI

Figure 6.4 Changes in plasma histidine concentration in individual horses (n = 6) following bolus intra-venous carnosine injection.

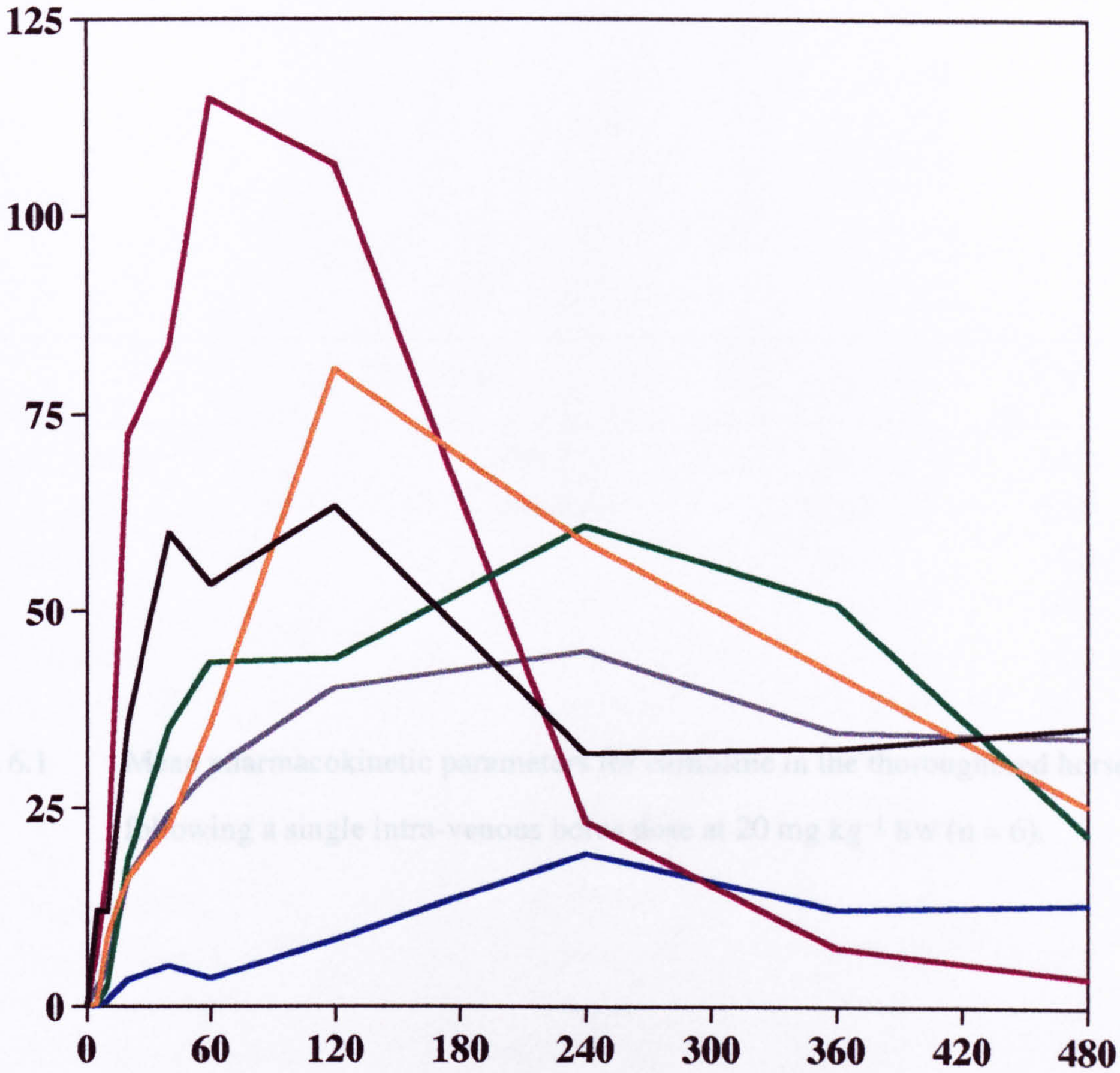
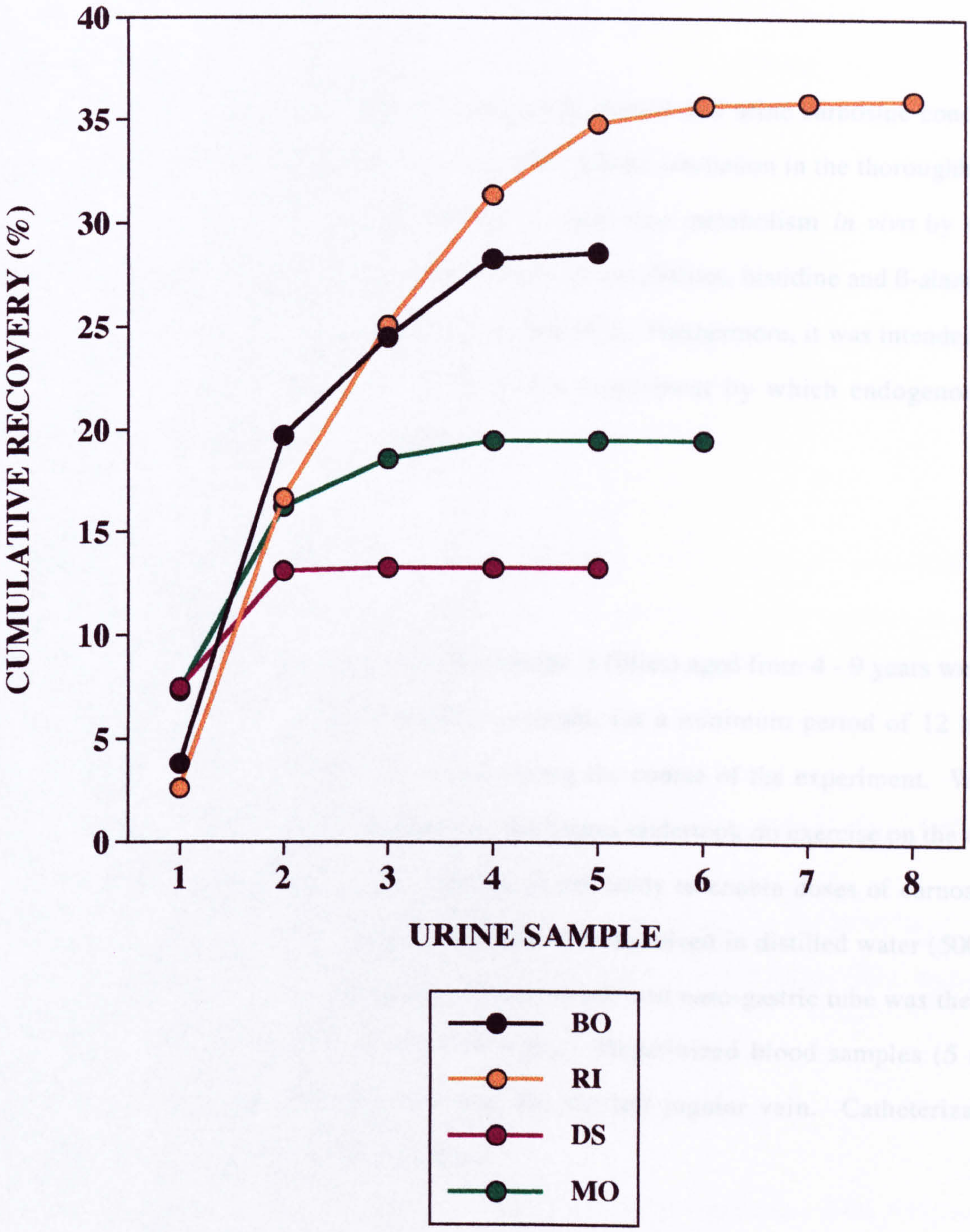

125
100
75
50
25
0
PLASMA HISTIDINE (μM)
0
60
120
180
240
300
360
420
480
TIME (min)
BR
DS
MO
GO
BO
RI

Table 6.1 Mean pharmacokinetic parameters for carnosine in the thoroughbred horse following a single intra-venous bolus dose at 20 mg kg^{-1} BW (n = 6).

	Mean	SD	CV
C_o (μM)	688.2	96.4	14.0
A (μM)	567.4	125.7	22.2
B (μM)	120.8	41.1	34.0
α (min^{-1})	0.0205	0.0021	10.0
ß (min^{-1})	0.0045	0.0010	22.4
k_{21} (min^{-1})	0.0073	0.0019	26.0
k_{10} (min^{-1})	0.0128	0.0016	12.9
k_{12} (min^{-1})	0.0050	0.0008	16.0
V_c ($l\ kg^{-1}$)	0.1308	0.0205	15.6
$V_{d(ss)}$ ($l\ kg^{-1}$)	0.2235	0.0234	10.5
$V_{d(area)}$ ($l\ kg^{-1}$)	0.3819	0.0858	22.5
CL ($l\ min^{-1}\ kg^{-1}$)	0.0017	0.0001	5.2
AUC ($\mu mol\ min\ l^{-1}$)	53797	2850	5.3
AUMC ($\mu mol\ min^2\ l^{-1}$)	7369167	1426627	19.4
MRT (min)	136.3	19.6	14.4
$t_{1/2\,(\alpha)}$ (min)	34.0	3.6	10.7
$t_{1/2\,(ß)}$ (min)	161.9	43.3	26.7

Figure 6.5 Cumulative percentage recovery of the administered carnosine dose in the urine of individual thoroughbred horses (n = 4) following a single intra-venous bolus injection at 20 mg kg^{-1} BW.


40
35
30
25
20
15
10
5
0
CUMULATIVE RECOVERY (%)
1
2
3
4
5
6
7
8
URINE SAMPLE
BO
RI
DS
MO

6.3 STUDY B: DETERMINATION OF CHANGES IN PLASMA CARNOSINE CONCENTRATION FOLLOWING ORAL ADMINISTRATION BY NASO-GASTRIC INTUBATION IN THE THOROUGHBRED HORSE.

6.3.1 Objectives

The aims of this study were to measure changes in plasma and urine carnosine concentrations following oral administration of carnosine by naso-gastric intubation in the thoroughbred horse. This was done in order to examine aspects of carnosine metabolism *in vivo* by measuring changes in the plasma and urine concentrations of its metabolites, histidine and ß-alanine, and to calculate the bio-availability of carnosine from the GIT. Furthermore, it was intended to assess the potential efficacy of carnosine for use as a supplement by which endogenous muscle carnosine concentration may be increased.

6.3.2 Experimental methodology

Protocol and sampling procedures

Nine experimental thoroughbred horses (6 geldings, 3 fillies) aged from 4 - 9 years were used in two groups of 5. All horses were fasted overnight, for a minimum period of 12 h, prior to undertaking the study and received no feed during the course of the experiment. Water was, however, provided *ad libitum* . Furthermore, the horses undertook no exercise on the day of the study. Horses were weighed on the morning of the study to enable doses of carnosine to be administered on a body weight basis. Carnosine was dissolved in distilled water (500 ml) and administered by naso-gastric intubation. The container and naso-gastric tube was then flushed through with a further 250 ml of distilled water. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration.

Carnosine was administered at three different doses. On the first occasion it was administered at 50 mg kg^{-1} BW (approximately 25 g total dose) to six horses, on the second occasion at 100 mg kg^{-1} BW (approximately 50 g total dose) to five horses of which 4 were the same as those used in

the first session, and on the final session at 200 mg kg^{-1} BW (approximately 100 g total dose) to six horses. Pre-administration blood samples were collected immediately prior to the carnosine administration, and subsequent blood samples were collected at 30, 60, 90 120, 180, 240, 360 and 480 min during the first two sessions, and additionally at 15, 45, 75, 105 and 300 min on the final (higher dose) session. Urine samples were collected from geldings only over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988).

Analytical methods

Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine ß-alanine concentrations were determined as described in Chapter 2.

Pharmacokinetic and statistical analysis

Pre-administration plasma concentrations were subtracted from all post-administration concentrations prior to calculating pharmacokinetic parameters. The area under the plasma carnosine concentration, Cp vs. time curve (AUC) from 0 - 480 min, following oral carnosine administration, was estimated using the Trapezoidal rule:

$$AUC_{0-480\,min} = \sum [1/2(C_0 + C_{15}]t_{0-15}\,,\, [1/2(C_{15} + C_{30})]t_{15-30}\,,\, \ldots\ldots\, [1/2(C_{360} + C_{480}]t_{360-480}$$

The bioavailability (F) of carnosine following oral administration, i.e. the percentage of the total administered dose which is absorbed and which enters the central compartment, was calculated according to:

$$F = 100(AUC_{oral} \,.\, Dose_{iv}/AUC_{iv} \,.\, Dose_{oral})$$

where AUC_{iv} and $Dose_{iv}$ values were taken from the preceding study. Both AUC and F were estimated for each individual horse using its corresponding AUC_{iv}, as determined in the preceding study, prior to calculating the mean value (± SD).

6.3.3 Results

Oral administration of carnosine by naso-gastric intubation, following overnight fasting, at doses of 50, 100 and 200 mg kg^{-1} BW resulted in significant increases in plasma carnosine concentrations, with mean (± SD) peak concentrations (C_{max}) of 20.6 ± 10.6, 54.7 ± 14.6 and 133.2 ± 40.1 μM, respectively. Peak concentrations occurred between 60 and 120 min after administration. Following administration at the highest dose, the plasma carnosine concentration at 480 min was still higher than the pre-administration value. Changes in plasma carnosine concentrations in individual horses following oral carnosine administration at doses of 50, 100 and 200 mg kg^{-1} BW are shown in Figure 6.6. Mean peak plasma carnosine concentrations increased proportionally with the increase in dose. A comparison of the changes in the mean (± SD) plasma carnosine concentrations following oral carnosine administration at the three doses are shown in Figure 6.7.

Increases in plasma histidine concentrations were also observed following oral carnosine administration. Peak plasma histidine concentration occurred at a similar time to the peak plasma carnosine concentration. Mean (± SD) plasma carnosine and histidine concentrations following oral administration at a dose of 200 mg kg^{-1} BW are shown in Figure 6.8. The mean (± SD) plasma C_{max}, t_{max}, AUC and F for carnosine and histidine following oral carnosine administration at the 3 doses are given in Table 6.2. There was a decrease in the proportion of the administered carnosine which was metabolized to histidine as the size of the dose was increased. ß-alanine was not detected in plasma or urine either before or after carnosine administration at any dose. No significant increases in urinary carnosine or histidine excretion were observed over 12 h following oral carnosine administration. The changes in urine plasma carnosine and histidine concentrations are shown in Figure 6.9.

Figure 6.6 Changes in plasma carnosine concentrations in individual horses following oral carnosine administration at doses of 50, 100 and 200 mg kg^{-1} BW. (n = 5 at 100 mg kg^{-1} BW, n = 6 at the other doses)

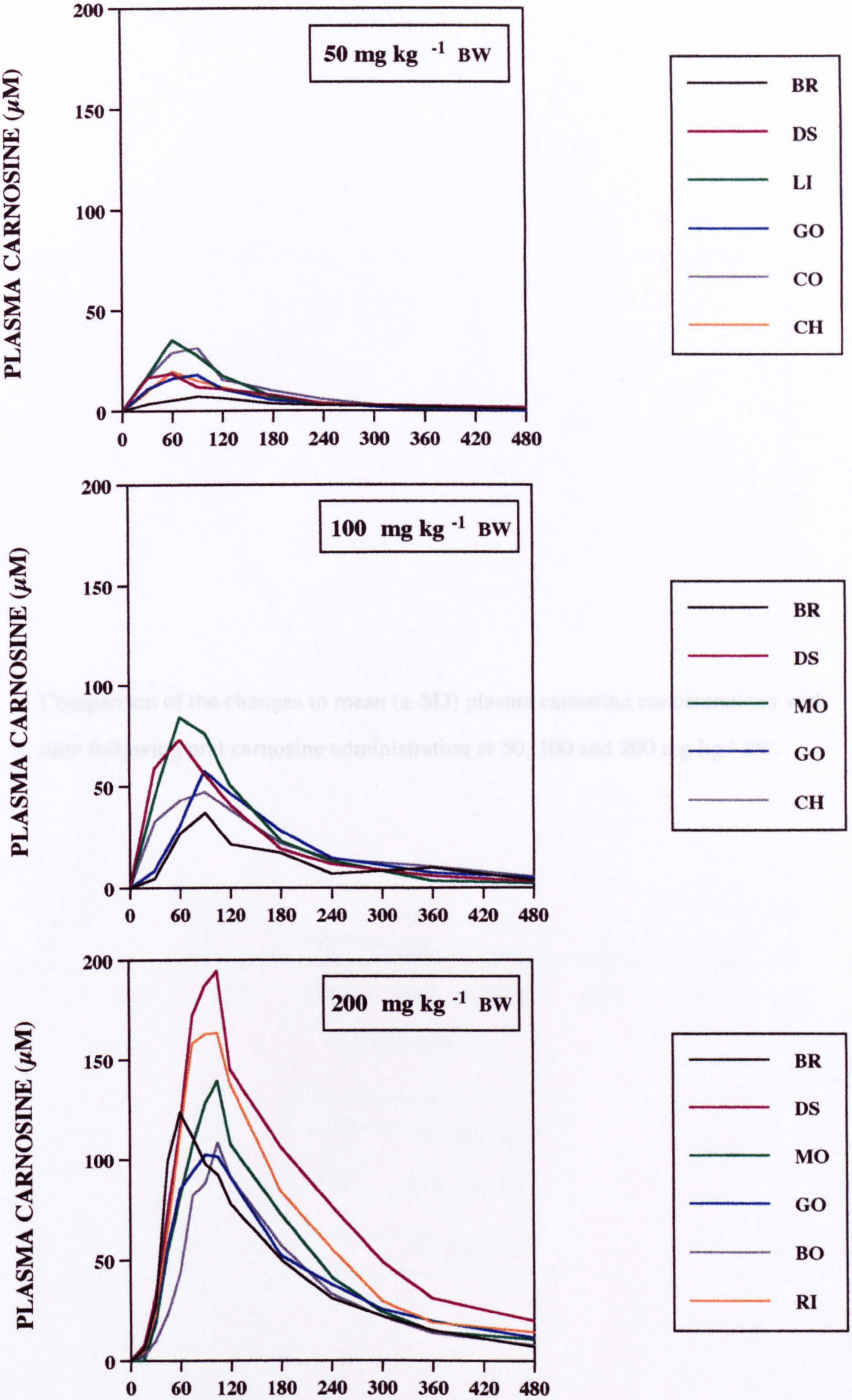

50 mg kg -1 BW
100 mg kg -1 BW
200 mg kg -1 BW
PLASMA CARNOSINE (μM)
TIME (min)
0
50
100
150
200
0 60 120 180 240 300 360 420 480
BR
DS
LI
GO
CO
CH
MO
BO
RI

Figure 6.7 Comparison of the changes in mean (± SD) plasma carnosine concentrations with time following oral carnosine administration at 50, 100 and 200 mg kg^{-1} BW.

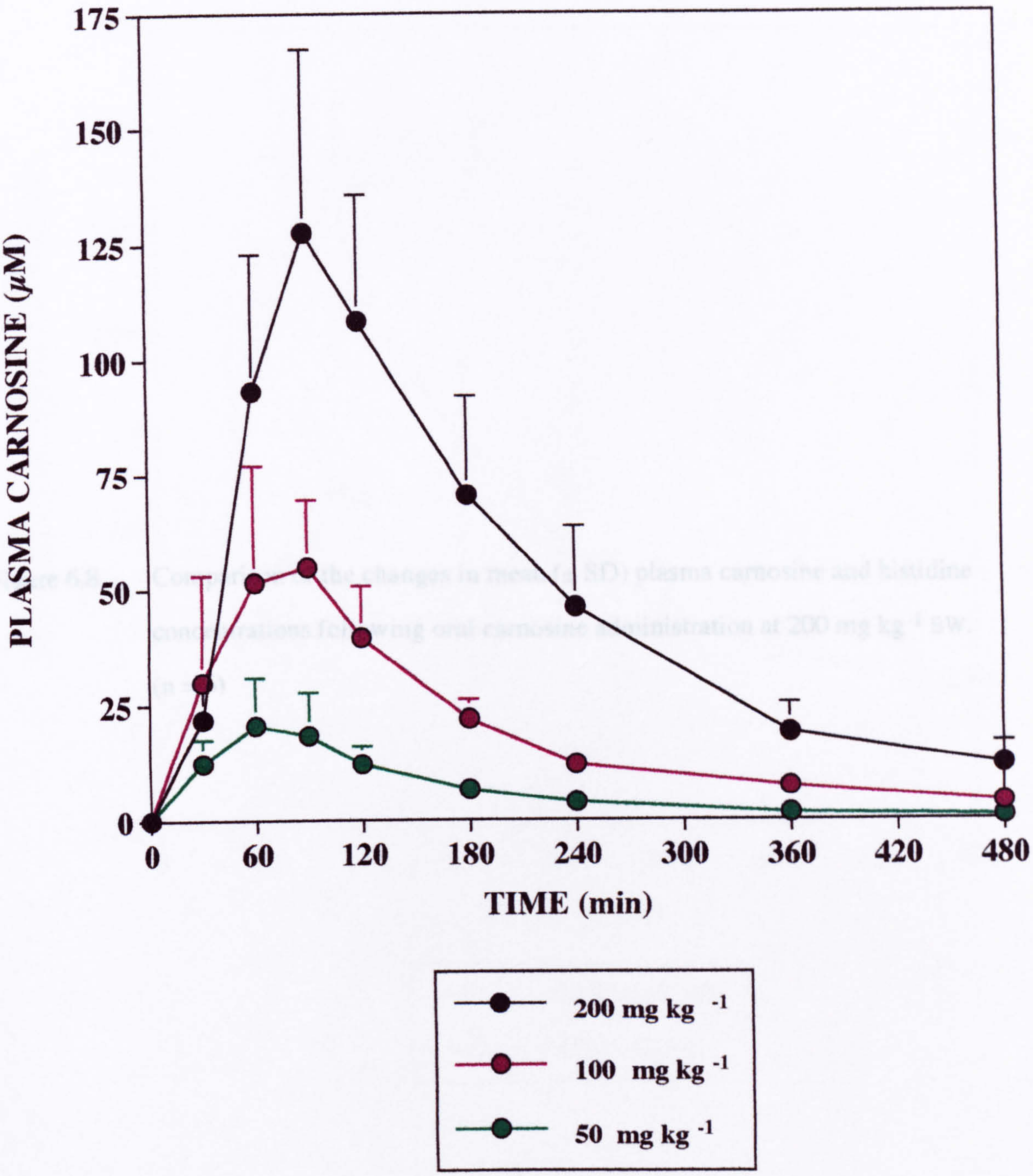

PLASMA CARNOSINE (μM)
175
150
125
100
75
50
25
0
0
60
120
180
240
300
360
420
480
TIME (min)
200 mg kg -1
100 mg kg -1
50 mg kg -1

Figure 6.8 Comparison of the changes in mean (± SD) plasma carnosine and histidine concentrations following oral carnosine administration at 200 mg kg^{-1} BW. (n = 6)

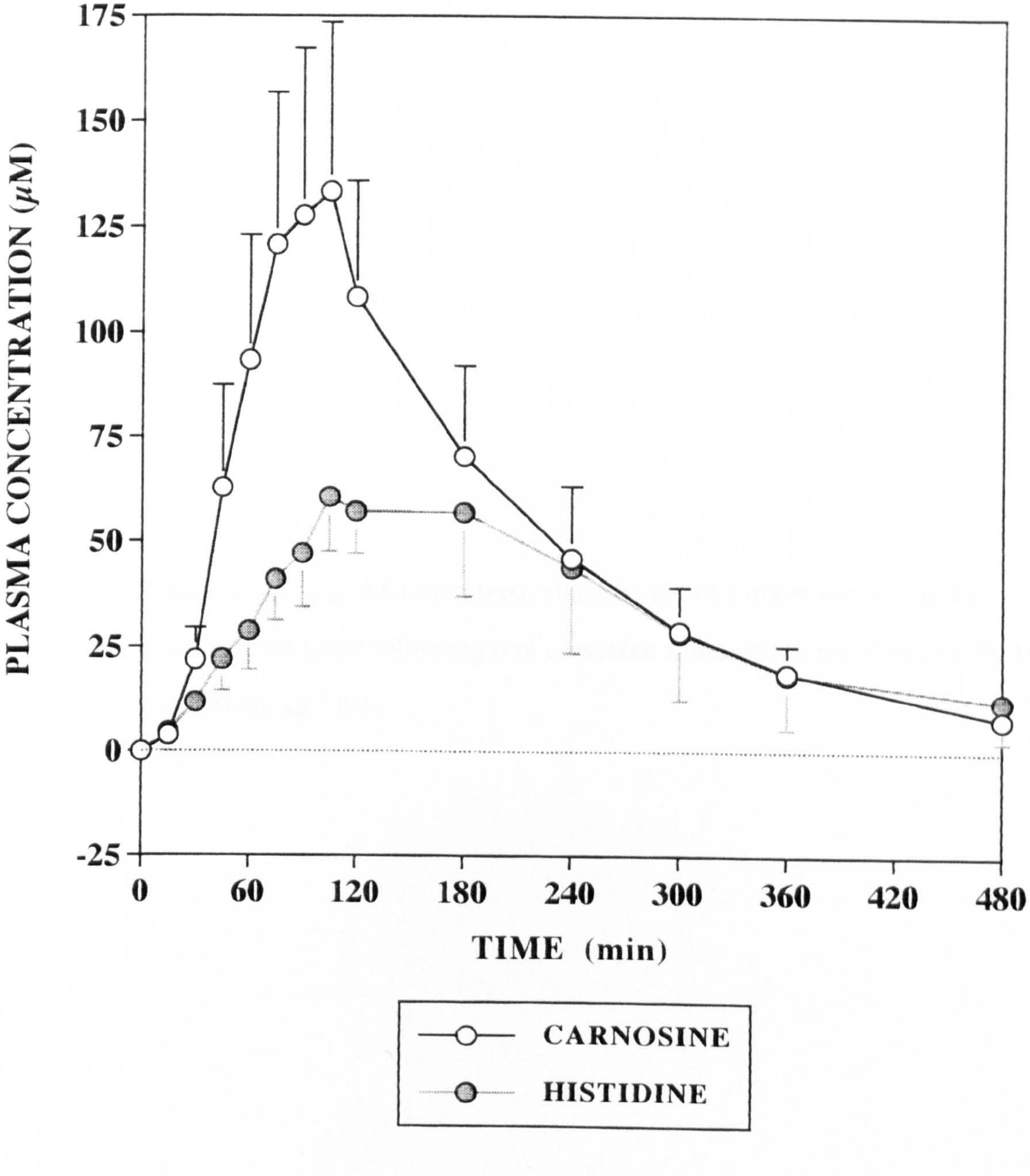
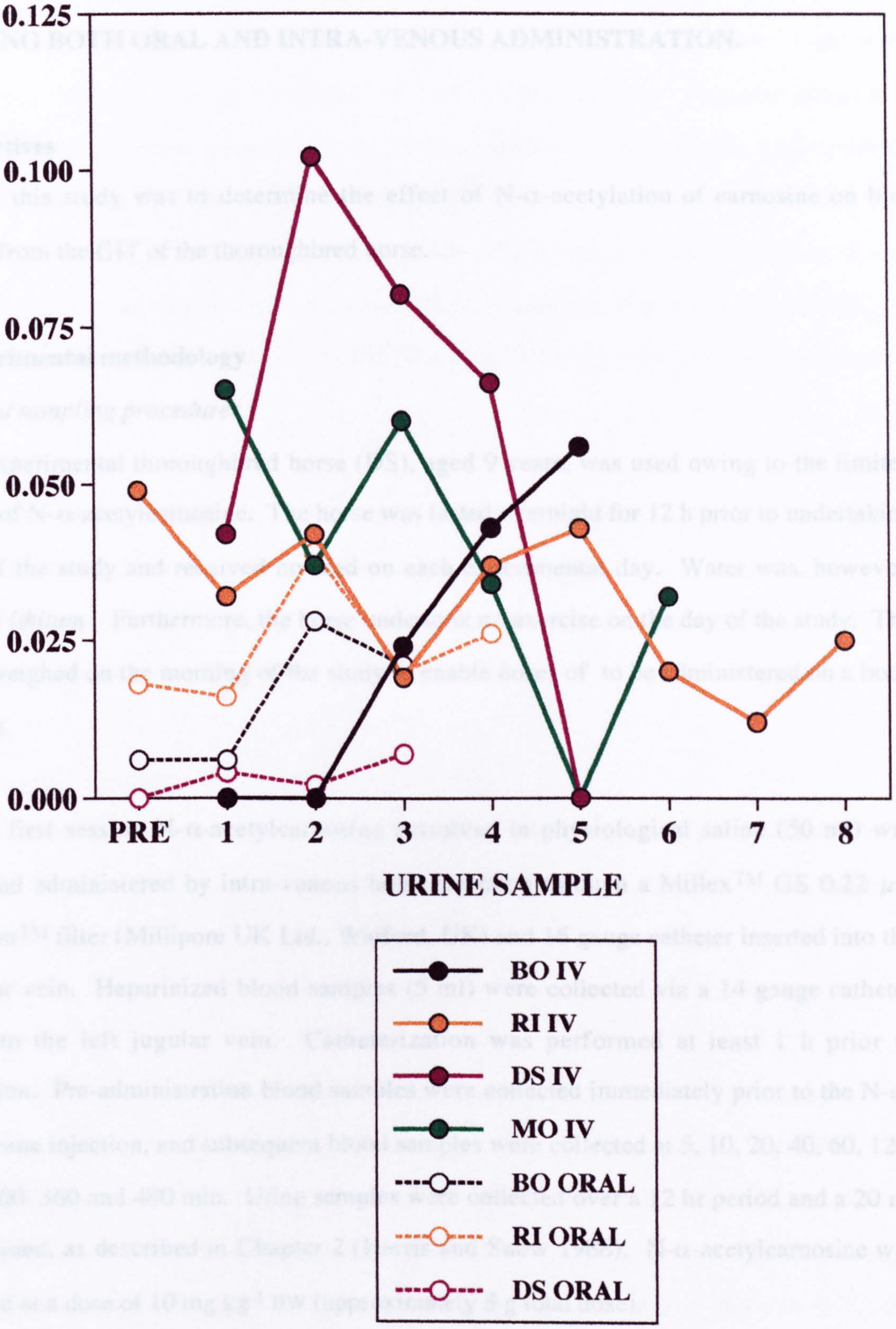

175
150
125
100
75
50
25
0
-25
PLASMA CONCENTRATION (μM)
0
60
120
180
240
300
360
420
480
TIME (min)
CARNOSINE
HISTIDINE

Table 6.2 Plasma C_{max}, t_{max}, AUC and bioavailability (F) of carnosine and histidine in the thoroughbred horse following oral carnosine administration at doses of 50, 100 and 200 mg kg^{-1} BW.

Carnosine dose	Metabolite	C_{max} (Mean ± SD)	t_{max}	AUC	Metabolism	F
		µM	min	µmol min l^{-1}	%	%
50 mg kg^{-1} BW	Carnosine	20.6 ± 10.3	60	3047 ± 1291	39	2.3 ± 1.0
	Histidine	20.1 ± 9.3	90	4764 ± 3839	61	
	(Total)	(40.7)		(7811)		
100 mg kg^{-1} BW	Carnosine	54.7 ± 14.6	90	9374 ± 3381	58	3.5 ± 1.3
	Histidine	29.0 ± 17.1	120	6717 ± 4880	42	
	(Total)	(83.7)		(16091)		
200 mg kg^{-1} BW	Carnosine	133.2 ± 40.1	105	23638 ± 7554	60	4.4 ± 1.4
	Histidine	60.5 ± 13.1	105	15464 ± 6350	40	
	(Total)	(193.7)		(39102)		

Figure 6.9 Comparison of histidine excretion in the urine of the thoroughbred horse following intra-venous and oral carnosine administration at 20 mg kg^{-1} BW and 200 mg kg^{-1} BW , respectively


0.125
0.100
0.075
0.050
0.025
0.000
URINE HISTIDINE (mmol)
PRE
1
2
3
4
5
6
7
8
URINE SAMPLE
BO IV
RI IV
DS IV
MO IV
BO ORAL
RI ORAL
DS ORAL

6.4 STUDY C: COMPARATIVE MEASUREMENTS OF CHANGES IN PLASMA N-α-ACETYLCARNOSINE CONCENTRATIONS IN THE THOROUGHBRED HORSE FOLLOWING BOTH ORAL AND INTRA-VENOUS ADMINISTRATION.

6.4.1 Objectives

The aim of this study was to determine the effect of N-α-acetylation of carnosine on bio-availability from the GIT of the thoroughbred horse.

6.4.2 Experimental methodology

Protocol and sampling procedures

Only one experimental thoroughbred horse (DS), aged 9 years, was used owing to the limited availability of N-α-acetylcarnosine. The horse was fasted overnight for 12 h prior to undertaking each part of the study and received no feed on each experimental day. Water was, however, provided *ad libitum* . Furthermore, the horse undertook no exercise on the day of the study. The horse was weighed on the morning of the study to enable doses of to be administered on a body weight basis.

During the first session N-α-acetylcarnosine dissolved in physiological saline (50 ml) was sterilized and administered by intra-venous bolus injection through a Millex^{TM} GS 0.22 μm sterile Teflon^{TM} filter (Millipore UK Ltd., Watford, UK) and 16 gauge catheter inserted into the right jugular vein. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration. Pre-administration blood samples were collected immediately prior to the N-α-acetylcarnosine injection, and subsequent blood samples were collected at 5, 10, 20, 40, 60, 120, 180, 240, 300, 360 and 480 min. Urine samples were collected over a 12 hr period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988). N-α-acetylcarnosine was administered at a dose of 10 mg kg^{-1} BW (approximately 5 g total dose).

During the second session N-α-acetylcarnosine was dissolved in distilled water (500 ml) and administered by naso-gastric intubation. The container and naso-gastric tube were flushed through with a further 250 ml of distilled water. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration. Pre-administration blood samples were collected immediately prior to the N-α-acetylcarnosine administration, and subsequent blood samples were collected at 30, 60, 90 120, 180, 240, 300, 360 and 480 min. Urine samples were collected over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988). N-α-acetylcarnosine was administered at a dose of 50 mg kg^{-1} BW (approximately 30 g total dose).

Analytical methods

Plasma N-α-acetylcarnosine concentration was determined by the HPLC method described in Chapter 3. Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine ß-alanine concentrations were determined, as described in Chapter 2.

Pharmacokinetic analysis

The determination of the mathematical equation describing the plasma N-α-acetylcarnosine concentration vs. time curve and the subsequent estimation of the conventional pharmacokinetic parameters were performed as described in Study A.

6.4.3 Results

Intra-venous bolus injection of N-α-acetylcarnosine produced a rapid increase in plasma N-α-acetylcarnosine to 296 μM 5 min after administration. This was followed by a rapid decline in the plasma N-α-acetylcarnosine concentration which reached the pre-administration value (1.9 μM) within 360 min. There was subsequently, only a very small increase in plasma carnosine concentration from a pre-administration value of 13.8 μM to a peak value of 20.4 μM at 20 min, but no increase in plasma histidine concentration. No ß-alanine was detected in the plasma following the injection of N-α-acetylcarnosine. The clearance of N-α-acetylcarnosine from

equine plasma following bolus intra-venous injection was described mathematically by the biexponential equation:

$$C_p = 165.4e^{-0.0426t} + 170.5e^{-0.0092t}$$

Pharmacokinetic parameters associated with a single bolus intra-venous injection of N-α-acetylcarnosine in the thoroughbred horse are given in Table 6.3.

Following oral administration of N-α-acetylcarnosine by naso-gastric intubation, no increase in plasma N-α-acetylcarnosine was observed, nor were there any increases in either plasma carnosine, histidine or ß-alanine concentrations over the subsequent 480 min. The urinary excretion of N-α-acetylcarnosine was not determined.

Table 6.3 Pharmacokinetic parameters for N-α-acetylcarnosine following a single intra-venous bolus injection at a dose 20 mg kg^{-1} BW.

Parameter	Value
C_o (μM)	335.9
A (μM)	165.4
B (μM)	170.5
α (min^{-1})	0.0426
ß (min^{-1})	0.0092
k21 (min^{-1})	0.0261
k10 (min^{-1})	0.0149
k12 (min^{-1})	0.0107
V_c ($l\ kg^{-1}$)	0.2220
$V_{d(ss)}$ ($l\ kg^{-1}$)	0.3127
$V_{d(area)}$ ($l\ kg^{-1}$)	0.3617
CL ($l\ min^{-1}\ kg^{-1}$)	0.0033
AUC ($\mu mol\ min\ l^{-1}$)	22485
AUMC ($\mu mol\ min^2\ l^{-1}$)	2119844
MRT (min)	94.3
$t_{1/2\,(\alpha)}$ (min)	16.3
$t_{1/2\,(ß)}$ (min)	75.6

6.5 DISCUSSION

No previous studies of carnosine transport and metabolism have been conducted following carnosine administration by intra-venous injection in any species. Following bolus intra-venous injection the change in plasma carnosine concentration (C_p) with time (t) in the thoroughbred horse was described adequately by the general biexponetial equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B are the zero time concentrations for the component distribution and elimination phases, and α and ß are the distribution and elimination rate constants, respectively. Therefore, data describing the change in plasma concentration following intra-venous bolus injection conformed to a two compartment model.

The mean $V_{d(ss)}$ value of 0.2235 l kg^{-1} for carnosine determined in Study A is equivalent to a total volume of approximately 112 l in a typical 500 kg horse, in contrast to an approximate total blood volume of 45 l. If an administered substance is extensively bound within the tissues then the $V_{d(ss)}$ will be greater than the volume of the total body water which in man is approximately 42 l or 0.6 l kg^{-1} (Hladky 1990). Assuming a similar volume of total body, water expressed per kg body weight in the horse then a $V_{d(ss)}$ of approximately 0.2 l kg^{-1} suggests that a significant amount of the administered carnosine is distributed to the extra-cellular fluid, but that little if any is bound within the tissues.

The fate of carnosine following intra-venous administration therefore appears to involve both direct excretion of the unmetabolized compound in the urine, which accounts for a mean of 11.76 ± 4.48 mmol or 24.4 ± 10.0% of the total administered dose, and catabolism to histidine catalysed by carnosinase and non-specific dipeptidase. There was however, a large between-horse variability in the amount of carnosine excreted in the urine. The proportion of the administered carnosine dose which is catabolized to histidine is difficult to quantify accurately in the absence of pharmacokinetic data describing both the distribution of histidine to the tissues and the elimination of histidine from the central compartment (including the plasma) following

intra-venous administration. If it is assumed that the overall distribution and elimination rate constants, α and ß, for histidine are very similar to those of carnosine, then for a given dose the AUC for histidine and carnosine would be the same. Significant increases in plasma histidine concentration following the intra-venous administration of carnosine at a dose of 20 mg kg^{-1} BW were seen, although there was considerable between-horse variation. Using the trapezoidal rule it was estimated that the mean $AUC_{0-480\ min}$ for histidine was 17950 μmol min ml^{-1} which would be equivalent to approximately 14.7 mmol, or 33% of the total carnosine dose administered. Over the same time period the mean total amount of histidine excreted in the urine was negligible, although it is possible that some of the histidine may have been deaminated via histidase and excreted in the urine as urocanic acid, or metabolized by other pathways (see Figures 1.3 and 1.4). In total urinary carnosine excretion and measured plasma histidine up to 480 min accounted for approximately 57% of the administered dose. The remaining portion of the administered dose was probably accounted for partly by elevated plasma histidine concentration beyond 480 min and histidine uptake by tissues. This data when considered together with the moderate value for the apparent $V_{d(ss)}$ suggests therefore, that there is minimal uptake, either by passive diffusion or active transport, of carnosine by low blood-flow equine tissues, such as skeletal muscle, *in vivo*. Nutzenadel *et al.* (1984) reported that there was no active uptake of carnosine in rabbit skeletal muscle *in vitro* , although the opposite was found in kidney. The rate of *in vivo* metabolism of carnosine to histidine in the thoroughbred horse following intra-venous administration was also highly variable between horses, which suggests a large between-horse variability in the activities of tissue carnosinase and non-specific dipeptidase, as described earlier in Chapter 4.

Although the bioavailability of carnosine following oral administration increased proportionally with increasing dose it was still very low, with only 4.5% of the total administered dose being absorbed at the highest dosage. The low bioavailability is probably due to the zwitterionic nature of carnosine, and hence an inability to penetrate lipid membranes. The fact that some increase in plasma carnosine occurs may be evidence for the existence of a specific, but low capacity, carnosine transporter in the equine GIT, as reported in other mammalian species, such as the

hamster (Matthews *et al.* 1974), rat (Nutzenadel and Scriver 1976), rabbit (Ganapathy and Leibach 1983), mouse (Rajendran *et al.* 1984) and guinea pig (Himuki 1985). This would however, seem to be unlikely given the absence of carnosine from the normal equine diet. More probably, some carnosine may be taken up via another amino acid or peptide transporter.

A portion of the carnosine dose is catabolized and appears in the plasma as histidine. At the highest dose level, where blood sampling was more frequent, the peak plasma carnosine and histidine concentrations occurred at the same time. This suggests that there is an initial phase of carnosine degradation in the GIT. Although a first pass effect through the liver cannot be excluded, data in Chapter 4 indicates a much higher carnosinase and non-specific dipeptidase activity in the GIT in comparison with the liver. From a comparison of the AUC for plasma carnosine and histidine it is probable that histidine accounts for a further 2 - 3% of the total dose absorbed at the highest dosage. The ratio of histidine to carnosine in the plasma decreases as the size of the dose increases, suggesting saturation of carnosinase and non-specific dipeptidase. This is consistent with the results from oral carnosine administration in the rat (Tamaki *et al.* 1985). Negligible excretion of carnosine and histidine was observed following oral carnosine administration in the horse, unlike that seen in humans (Gardner *et al.* 1991). This probably reflects the small quantities which enter the systemic circulation and the longer interval over which this takes place, in contrast to intra-venous administration.

The absence of N-α-acetylcarnosine in plasma following its oral administration suggests that the free terminal amine moiety of carnosine is essential for its uptake from the GIT, although carnosine uptake is low normally.

Increased plasma activities of cytosolic muscle enzymes, such as AST and CK, are associated with changes in skeletal muscle cell membrane permeability, or cell damage (Janssen *et al.* 1989). This can occur as a result of intra-muscular injection in humans (Metzler *et al.* 1970) and dogs (Aktas *et al.* 1995), exercise in humans (Noakes 1987; Janssen *et al.* 1989) and horses (Anderson 1975; Rose *et al.* 1983) and ERS in horses (Cardinet *et al.* 1963; Harris 1989). The

increase in plasma AST and CK activity has been used to estimate the mass of muscle damaged during trauma (Lefebvre *et al.* 1994) or following exercise in humans (Appel and Rhodes 1988; Janssen *et al.* 1989) and horses (Volfinger *et al.* 1994). However, AST and CK are not specific to skeletal muscle. Significant amounts of AST are present in the liver and heart, and CK in the heart and brain. Other factors cause an increase in plasma CK activity, such as cardiac muscle damage associated with rhabdomyolysis (Fujii *et al.* 1983), myocardial infarction (Klein *et al.* 1973), and incorrect venepuncture technique (Fayolle *et al.* 1992) can lead to the erroneous diagnosis of skeletal muscle damage, or to an over-estimation of the mass of muscle damaged. Furthermore, examination of skeletal muscle by electron microscopy indicates less extensive damage than is suggested by parallel measurement of plasma enzyme activity (van-der-Meulen *et al.* 1991). Carnosine is almost exclusively distributed within the skeletal muscles, with concentrations in other tissues, such as the heart and GIT being 50 to 100-fold lower than that in skeletal muscle. Consequently, a large increase in plasma carnosine concentration would provide a much more specific indicator of skeletal muscle damage than AST or CK.

The controlled intra-venous bolus injection of carnosine in the horse could be used as a simple model to represent the changes in plasma carnosine concentrations following the onset of muscle damage during episodes of ERS, as described in the preceding chapter. The area under the concentration-time curve (AUC) following an intra-venous bolus injection is proportional to the total dose administered. Therefore the AUC calculated from the change in plasma carnosine concentration following release from damaged muscle would be proportional to the total amount released. Hence, the AUC from a known intra-venous dose could be used to calculate the mass of carnosine released into the circulation from damaged skeletal muscle, and hence an estimate of the mass of muscle damaged.

CHAPTER 7

EFFECT OF DIETARY SUPPLEMENTATION WITH L-HISTIDINE AND β-ALANINE ON CARNOSINE CONCENTRATIONS IN TYPE I, IIA AND IIB MUSCLE FIBRES OF THE MIDDLE GLUTEAL OF THE THOROUGHBRED HORSE

7.1 INTRODUCTION

Dietary availability of histidine plays an important role in the regulation of the biosynthesis and accumulation of carnosine in vertebrate skeletal muscle. The effects of both histidine deficient diets and histidine supplemented diets have been investigated in several species. Reduced skeletal muscle carnosine concentrations were found in experimental adult rats (Fuller *et al.* 1947; Quinn and Fisher 1977), eels (Abe and Ohmama 1987) and salmon (Luckton 1958) maintained on a histidine deficient diet in contrast to controls. A similar reduction in the concentration of carnosine and anserine in the breast and leg muscles of adult roosters maintained on a histidine-free diet for a two week period has also been reported (Leveille *et al.* 1960). Amend *et al.* (1979) reported that pectoral and leg muscle carnosine and anserine concentrations in adult cockerels, depleted by 12 weeks maintenance on histidine deficient diets, were subsequently restored to normal levels after 4 weeks on a histidine supplemented diet. Dietary histidine deficiency causes a more rapid decline in muscle carnosine concentration in younger animals. A decline in chick pectoral muscle carnosine concentration to 4% of its initial concentration following 5 weeks of histidine deprivation has been reported (Ousterhout 1960; Ousterhout and Luckton 1960). Histidine deprivation also causes a more rapid decline in skeletal muscle carnosine concentration in younger rats (Barbaro *et al.* 1978) than in older rats (Barbaro *et al.* 1977; Tamaki *et al.* 1977). Furthermore, other studies indicate that histidine is an essential dietary amino acid during the growing phase in other species including the human infant (Snyderman *et al.* 1963; Strecker 1970), although 24 days of histidine deprivation in young pregnant sows caused no significant reduction in muscle carnosine concentrations (Easter and Baker 1977).

It has been shown by providing increasing levels of histidine in the diet, that increases in the skeletal muscle carnosine concentration occur once the histidine requirements for optimum growth have been fulfilled (Robbins *et al.* 1977). Rats fed a diet supplemented with histidine at a level of 5% w/w showed a two-fold greater carnosine concentration in the gastrocnemius muscle in contrast to rats on a control diet with a histidine content of 0.76% w/w (Tamaki *et al.* 1977). In the mature Quarterhorse, supplementation of a basal diet with histidine, at levels of 0.4% w/w

over a period of two weeks in one study and up to 0.56% w/w twice per day in a further study, produced small but statistically non-significant increases in the carnosine concentration of the middle gluteal muscle (Powell *et al.* 1991; Miller-Graber and Seyers 1993).

In contrast to the extensive investigations of the influence of dietary histidine on skeletal muscle carnosine concentration, the effect of dietary ß-alanine supplementation on muscle carnosine content has not been investigated. However, Margolis (1981) demonstrated an increase in olfactory bulb carnosine concentration in adult mice following ß-alanine administration. Furthermore, twice-daily intra-peritoneal injections of very large doses of ß-alanine (22 mmol kg^{-1} BW, 2000 mg kg^{-1} BW) in adult mice over a 5 day period produced a ten-fold increase in skeletal muscle carnosine concentration in contrast to controls (Margolis *et al.* 1985).

The results of earlier investigations in other species suggest that substrate availability may be a limiting factor to the regulation of carnosine biosynthesis and accumulation within skeletal muscle. Thus increasing the endogenous concentrations of both histidine and ß-alanine may result in an increase in muscle carnosine content. Histidine and ß-alanine required for carnosine synthesis in the skeletal muscles are probably obtained via active transport from the blood. Earlier results show a normal plasma histidine concentration in the mature horse of approximately 50 - 60 μM (Chapter 5). ß-alanine was however, not detected in normal plasma indicating a concentration of less than 3 μM, which was the lower limit of quantification for the analysis (Chapter 5). The low plasma ß-alanine concentration may be due to the absence of significant quantities of ß-alanine from the normal equine diet and/or inadequate ß-amino acid membrane transport mechanisms in the gastro-intestinal tract (GIT). It is therefore possible that a large and sustained increase in plasma ß-alanine concentration in conjunction with a moderate increase in plasma histidine concentration in the horse, via dietary supplementation, may have a more significant effect on muscle carnosine biosynthesis than that previously achieved via histidine alone (Powell *et al.* 1991; Miller-Graber and Seyers 1993). This approach does not appear to have been adopted in earlier supplementation studies.

Increasing the carnosine concentration within the skeletal muscles of the horse will produce an increase in the total intra-cellular physico-chemical buffering capacity of the muscles and thus may attenuate the rate of decline in intra-muscular pH during anaerobic exercise such as sprinting and prolong the time to fatigue.

7.2 EFFECT OF DIETARY SUPPLEMENTATION WITH L-HISTIDINE AND ß-ALANINE ON CARNOSINE CONCENTRATIONS IN TYPE I, IIA AND IIB MUSCLE FIBRES OF THE MIDDLE GLUTEAL OF THE THOROUGHBRED HORSE.

7.2.1 Objectives

The aim of the study was to investigate whether chronic supplementation of the normal diet with multiple daily doses of L-histidine and ß-alanine would increase the carnosine content of type I, IIA and IIB skeletal muscle fibres.

7.2.2 Experimental methodology

Protocol and sampling procedure

Three experimental thoroughbred horses (DS, JS, GT) (2 fillies and one gelding) aged 4, 5 and 9 years, respectively, underwent one month of dietary conditioning (pre-supplementation period) prior to the commencement of the supplementation period. During the dietary conditioning phase each horse was fed a diet comprising 1 kg of pelleted feed (Spillers racehorse cubes) and 1 kg of soaked sugar beet pulp, three times per day, at 08.30 h, 12.30 h and 16.30 h, respectively. Soaked hay (3 kg dry weight) was also provided twice daily at 09.00 h and 17.00 h. Water was provided *ad libitum* .

During the supplementation period an identical feeding regime was implemented. However, each hard feed meal was supplemented with L-histidine (free base) and ß-alanine. Histidine and ß-alanine were administered as dry powders mixed directly into the normal feed. Individual doses of histidine and ß-alanine were calculated according to body weight. ß-alanine was administered at 100 mg kg^{-1} BW and histidine at 12.5 mg kg^{-1} BW. Dietary supplementation was

begun on day 1 of the protocol and discontinued at the end of day 30. From day 31 to day 60 the horses were returned to the non-supplemented diet used during the conditioning phase. Heparinized blood samples (5 ml) were collected on days 1, 6, 12, 18, 24 and 30. On days 1 and 30 blood samples were collected prior to the first feed and at hourly intervals thereafter up to 12 h. On the four intervening sampling days blood was collected prior to the first feed and 2 h after each subsequent feed. On day 0, pre-supplementation muscle samples were collected from the right middle gluteal muscle of each horse using the percutaneous needle biopsy technique described in Chapter 2. Subsequent muscle biopsies were collected immediately after the end of the supplementation period (day 31) and thirty days after returning to the non-supplemented diet (day 60). A diagrammatic representation of the experimental protocol and sampling intervals is shown in Figure 7.1. Clinical monitoring of the horses was performed daily over all three experimental periods. Clinical monitoring comprised a visual examination and measurement of body weight on a daily basis, twice-daily measurement of rectal temperature, and weekly blood sampling for clinical biochemistry and haematology. During the course of the study the horses received no formal training or exercise, although they were allowed one hour of free exercise each day.

Histochemistry

Fragments of individual muscle fibres were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described in Chapter 2.

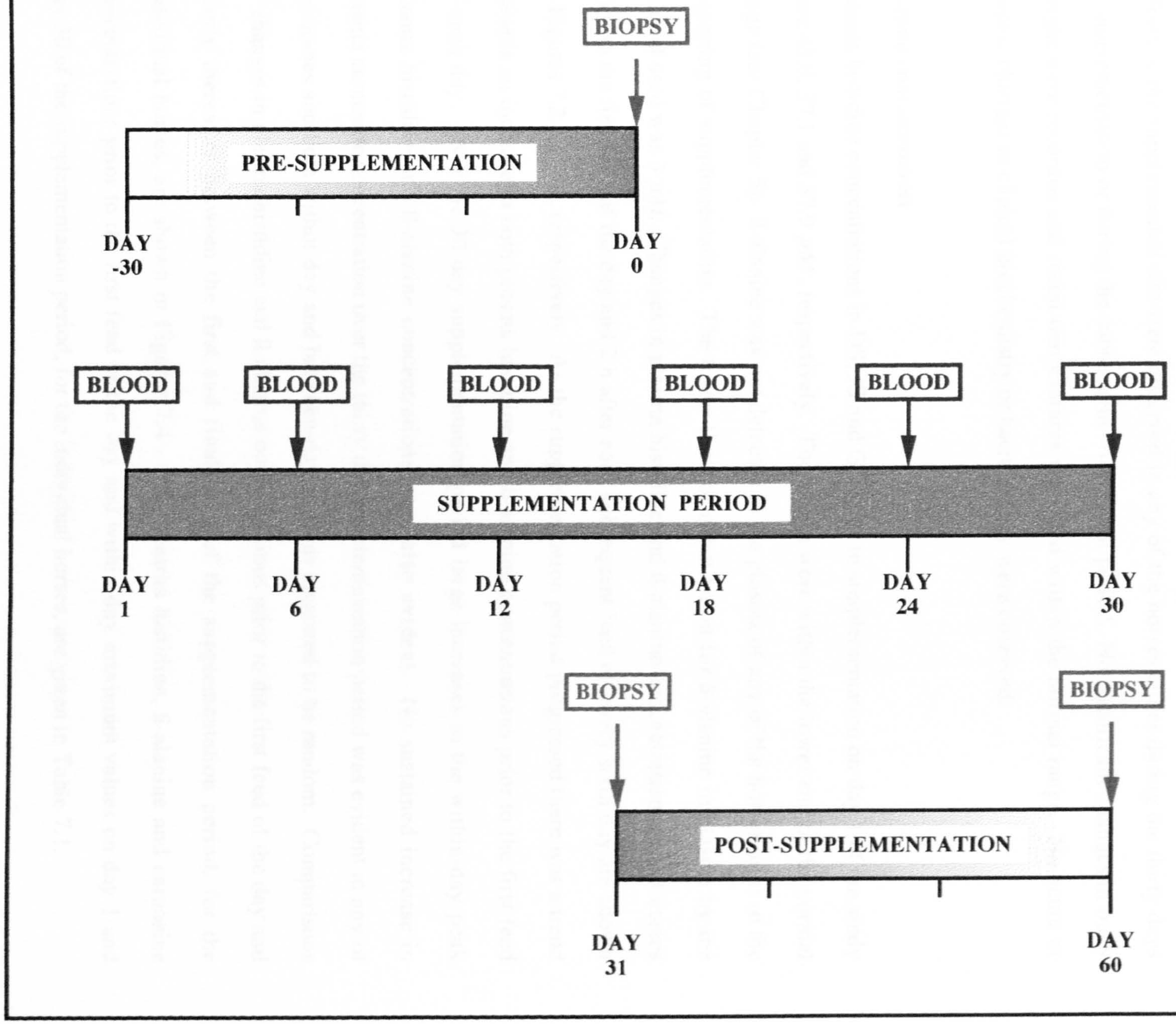
Plasma and individual muscle fibre analysis

Heparinized plasma samples were extracted and analysed for carnosine and histidine, and ß-alanine concentrations by the HPLC methods described in Chapter 3 (Dunnett and Harris 1992) and Chapter 2, respectively. Weighed individual muscle fibres were extracted and analysed for carnosine and taurine concentrations by the method described in Chapter 3 (Dunnett and Harris 1995b).

Statistical analysis

Owing to the small number of horses used in this study no between-horse statistical comparisons were made. Differences in carnosine and taurine concentrations within fibre types before and after supplementation were established within horses using 1-way ANOVA. In instances where differences were detected, significance was determined using a multiple comparison test (Fisher's PLSD).

Figure 7.1 Diagrammatic representation of the experimental protocol and sampling intervals.


BIOPSY
PRE-SUPPLEMENTATION
DAY
-30
DAY
0
BLOOD
BLOOD
BLOOD
BLOOD
BLOOD
BLOOD
SUPPLEMENTATION PERIOD
DAY
1
DAY
6
DAY
12
DAY
18
DAY
24
DAY
30
BIOPSY
BIOPSY
POST-SUPPLEMENTATION
DAY
31
DAY
60

7.3 Results

All three horses were clinically normal prior to commencing the supplementation. No palatability problems with the histidine and ß-alanine supplemented diet were encountered and complete feeds were consumed on every occasion. No adverse physiological or behavioural effects of the supplemented diet were observed in any of the horses either during the thirty days of supplementation or during the subsequent withdrawal period. No significant changes in body weight were recorded and rectal temperatures remained within the normal range. No acute or chronic changes in clinical biochemistry or haematology were observed.

Plasma concentrations

Plasma histidine concentrations in DS, JS and GT prior to supplementation on day 1 of the study were 43.8, 37.1 and 37.9 μM , respectively. The values were within the lower end of the normal range (see Chapter 5). ß-alanine was not detected in the plasma of any of the horses prior to the beginning of supplementation. The lower limit of quantitation for ß-alanine in plasma by the method used was 3 μM. Changes in plasma histidine and ß-alanine concentrations for all horses prior to the first feed of the day and 2 h after each subsequent feed on every sixth day are shown in Figures 7.2 and 7.3, respectively. As the supplementation period progressed there was a trend towards an increase in both plasma histidine and ß-alanine concentrations prior to the first feed of each day. Over the 30 day supplementation period large increases in the within-day peak plasma histidine and ß-alanine concentrations were also evident. No sustained increase in plasma carnosine concentration over the thirty day supplementation period was evident in any of the horses and both within-day and between-day changes appeared to be random. Comparisons of changes in plasma histidine and ß-alanine concentrations prior to the first feed of the day and hourly thereafter between the first and final days of the supplementation period, for the individual horses, are shown in Figures 7.4 - 7.6. Plasma histidine, ß-alanine and carnosine concentrations prior to the first feed of the day and within-day maximum values on day 1 and day 30 of the supplementation period, for the individual horses, are given in Table 7.1.

Figure 7.2 Changes in pre-feeding and 2 h post-feeding plasma histidine concentrations for the individual horses.

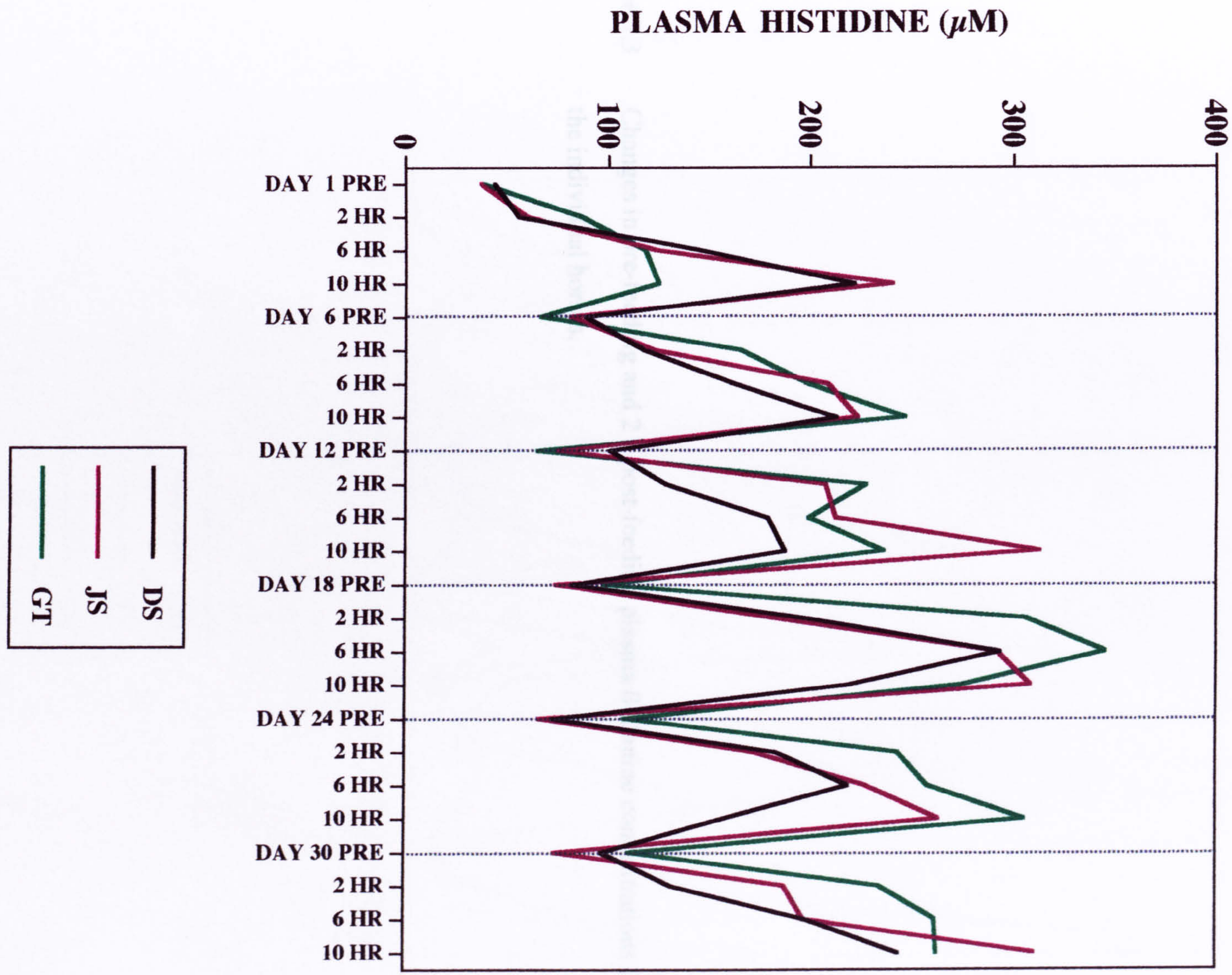

PLASMA HISTIDINE (μM)
0
100
200
300
400
DAY 1 PRE
2 HR
6 HR
10 HR
DAY 6 PRE
2 HR
6 HR
10 HR
DAY 12 PRE
2 HR
6 HR
10 HR
DAY 18 PRE
2 HR
6 HR
10 HR
DAY 24 PRE
2 HR
6 HR
10 HR
DAY 30 PRE
2 HR
6 HR
10 HR
GT
JS
DS

Figure 7.3 Changes in pre-feeding and 2 h post-feeding plasma ß-alanine concentrations for the individual horses.

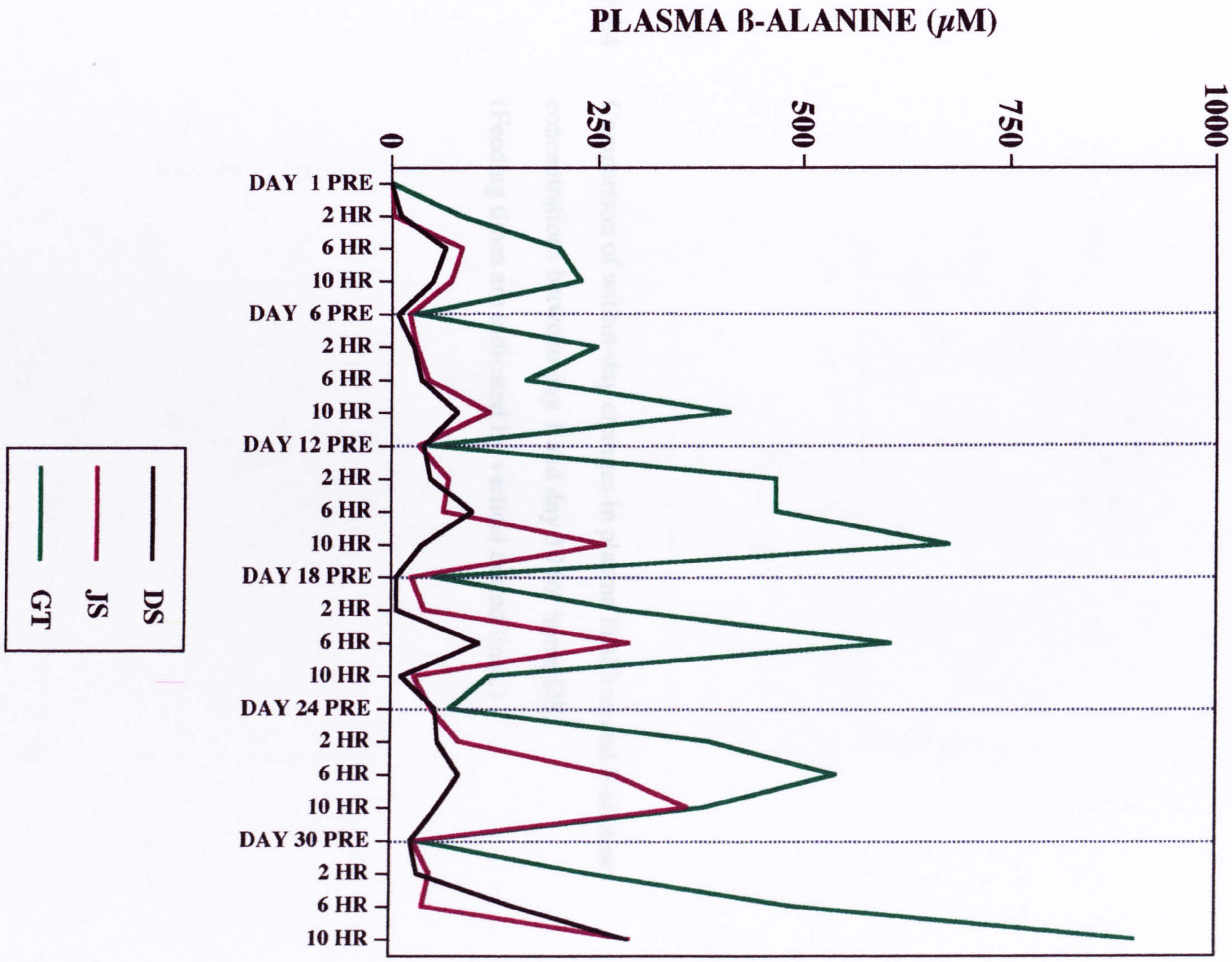
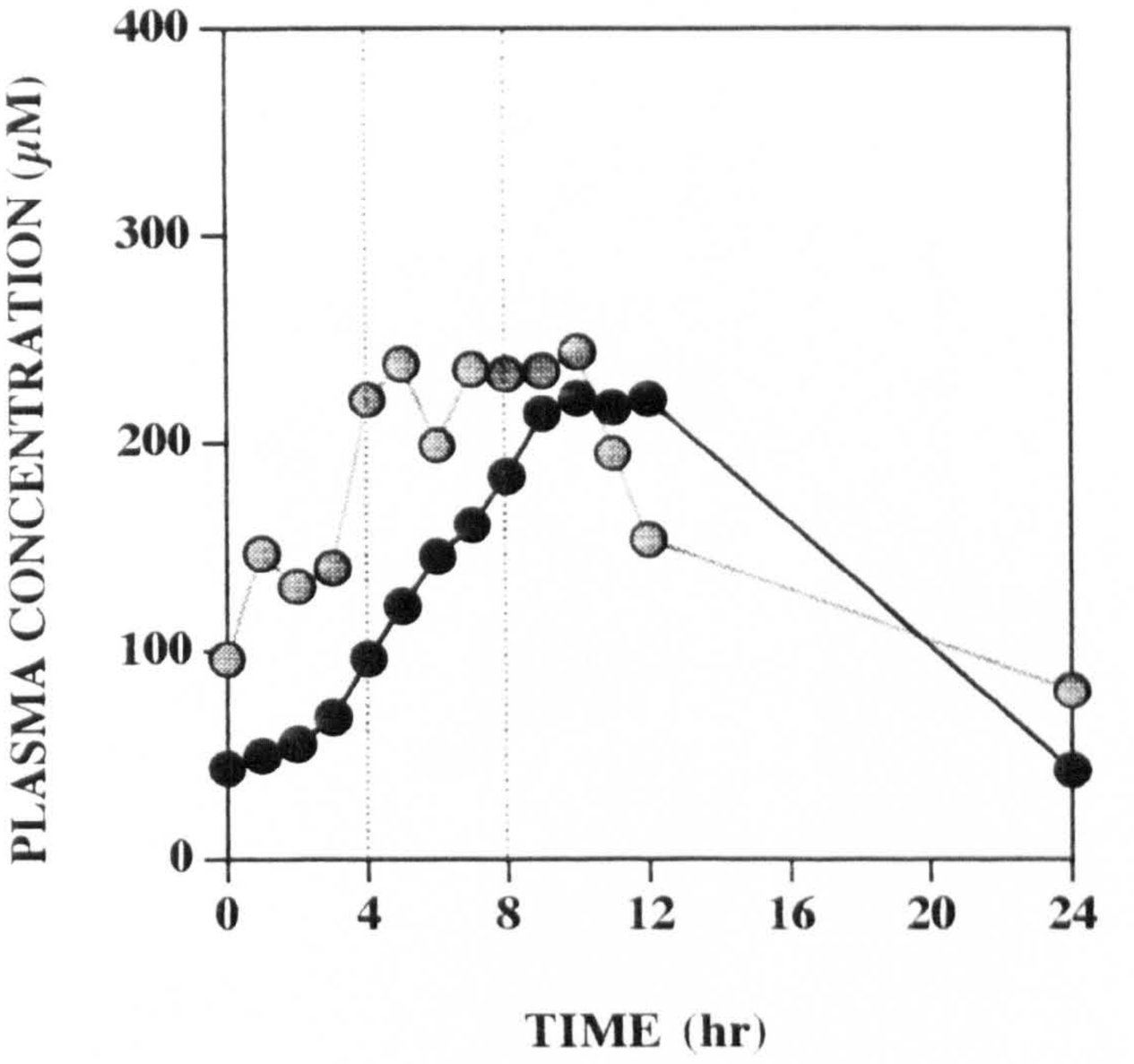

PLASMA ß-ALANINE (μM)
0
250
500
750
1000
DAY 1 PRE
2 HR
6 HR
10 HR
DAY 6 PRE
2 HR
6 HR
10 HR
DAY 12 PRE
2 HR
6 HR
10 HR
DAY 18 PRE
2 HR
6 HR
10 HR
DAY 24 PRE
2 HR
6 HR
10 HR
DAY 30 PRE
2 HR
6 HR
10 HR
GT
JS
DS

Figure 7.4 Comparison of within-day changes in plasma histidine and ß-alanine concentrations between day 1 and day 30 for horse DS.

(Feeding times are indicated by vertical dotted lines.)

HISTIDINE



ß-ALANINE

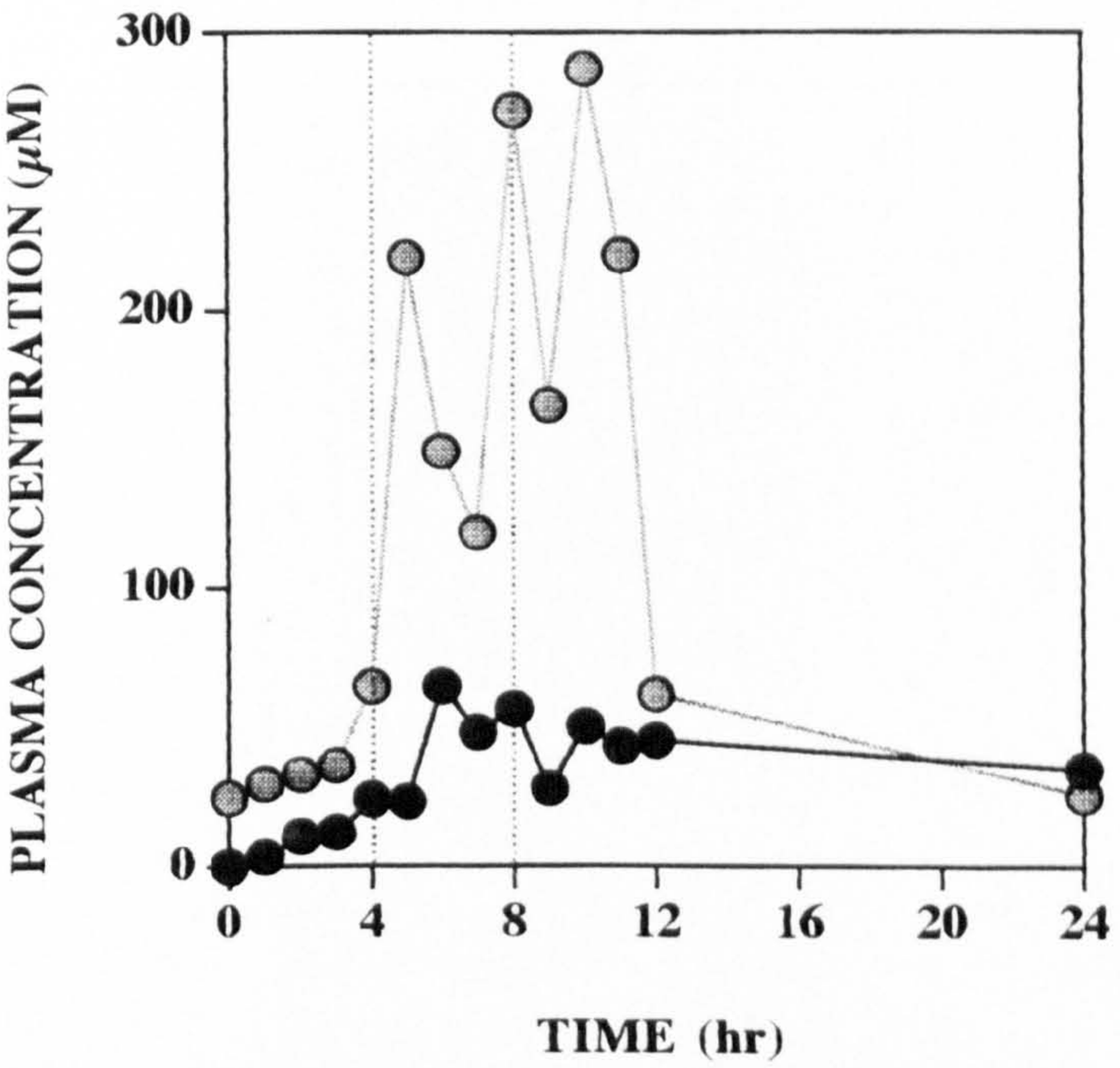
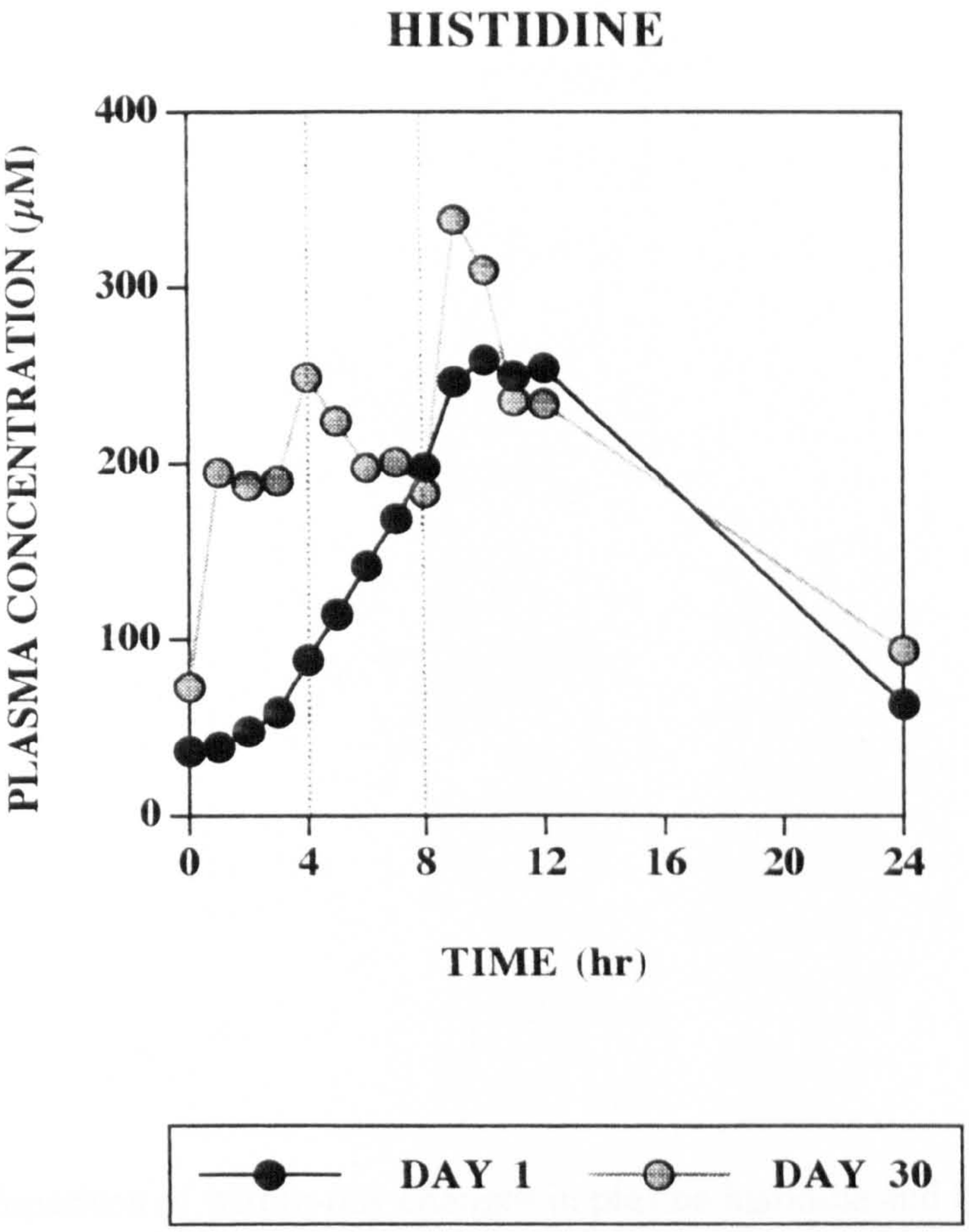


Figure 7.5 Comparison of within-day changes in plasma histidine and ß-alanine concentrations between day 1 and day 30 for horse JS.

(Feeding times are indicated by vertical dotted lines.)


HISTIDINE
PLASMA CONCENTRATION (μM)
400
300
200
100
0
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

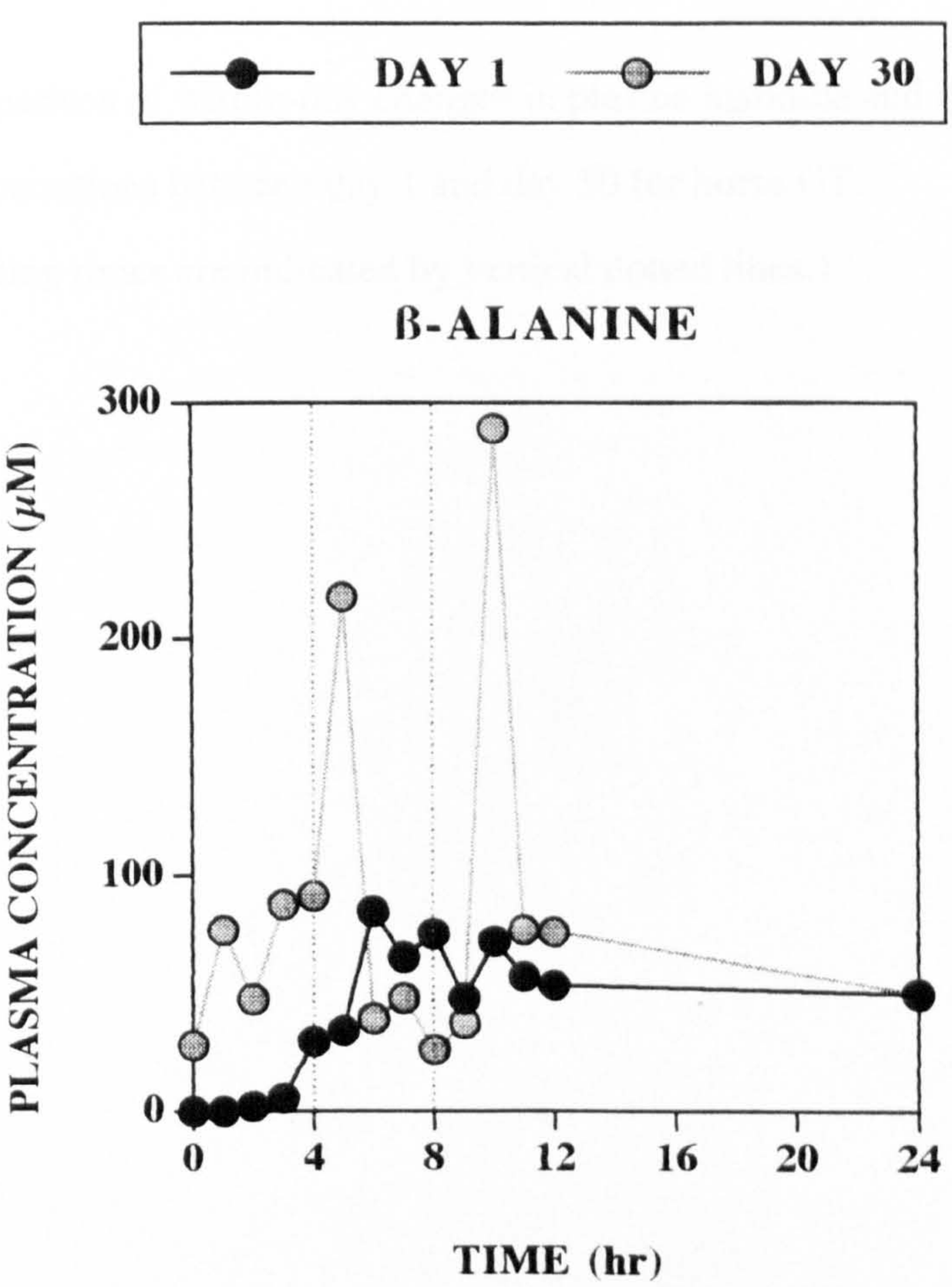
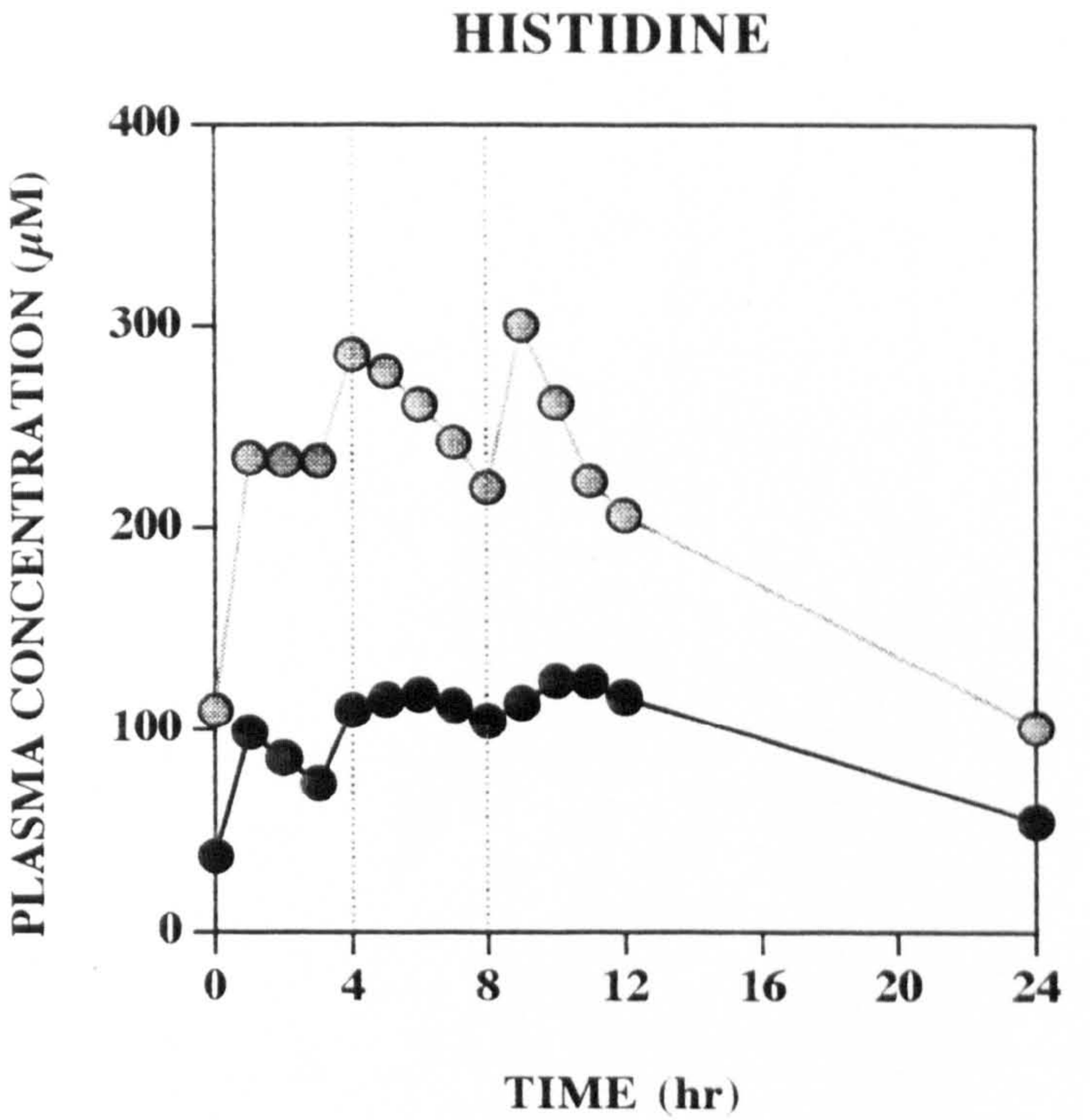

ß-ALANINE
PLASMA CONCENTRATION (μM)
300
200
100
0
0
4
8
12
16
20
24
TIME (hr)

Figure 7.6 Comparison of within-day changes in plasma histidine and ß-alanine concentrations between day 1 and day 30 for horse GT.

(Feeding times are indicated by vertical dotted lines.)


HISTIDINE
PLASMA CONCENTRATION (μM)
400
300
200
100
0
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

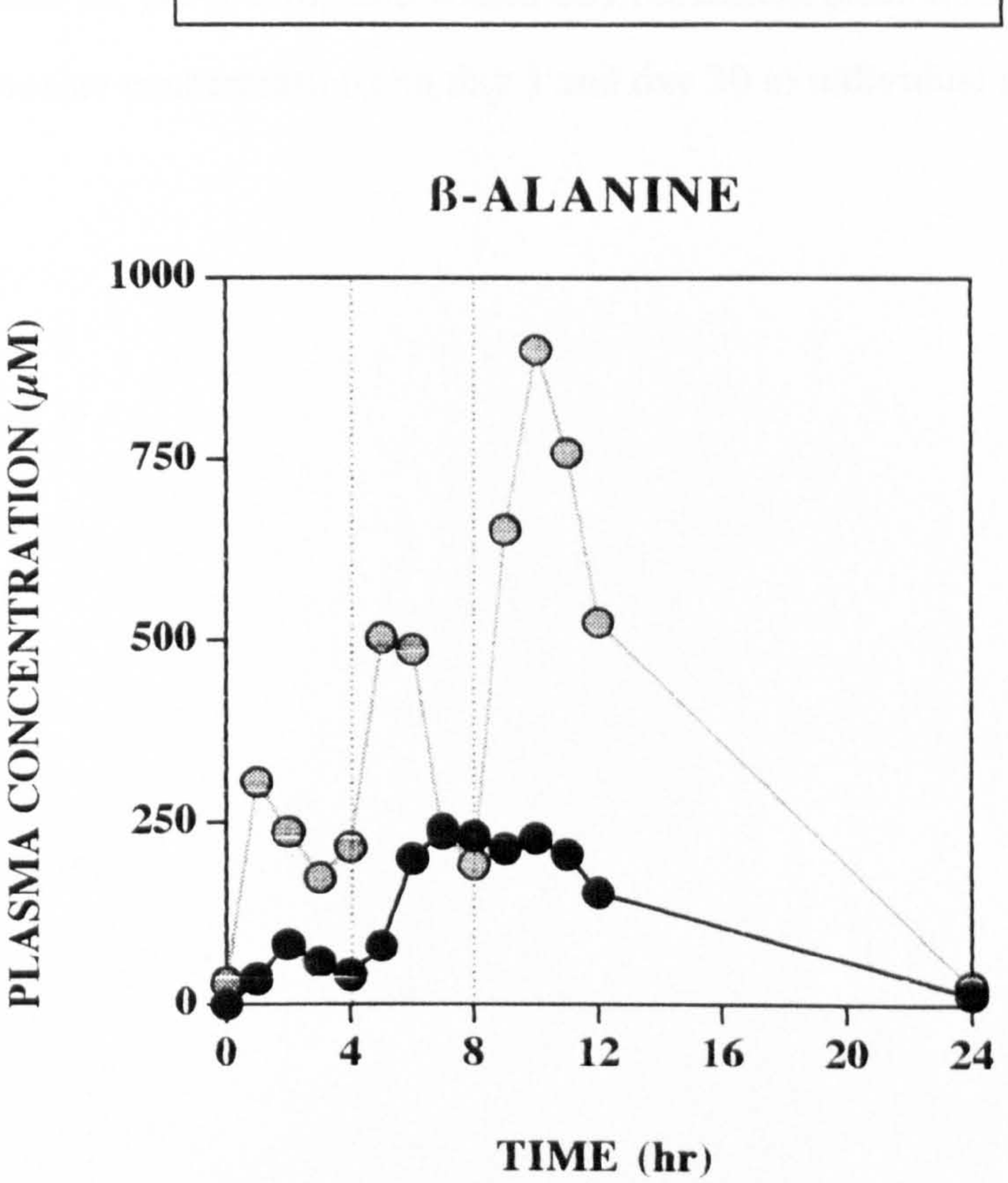

ß-ALANINE
PLASMA CONCENTRATION (μM)
1000
750
500
250
0
0
4
8
12
16
20
24
TIME (hr)

Table 7.1 Values for pre-feeding and within-day maximum plasma histidine, ß-alanine and carnosine concentrations on day 1 and day 30 in individual horses.

Horse	Value	Plasma histidine (μM)		Plasma ß-alanine (μM)		Plasma carnosine (μM)	
		Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
DS	Pre-feeding	43.8	96.2	0.0	24.6	16.6	15.6
	Within-day maximum	221.2	243.6	64.5	286.9	16.6	18.0
JS	Pre-feeding	37.1	73.5	0.0	28.2	12.5	14.2
	Witnin-day maximum	258.7	338.6	84.8	289.1	12.5	14.9
GT	Pre-feeding	37.9	109.8	0.0	30.0	13.3	15.7
	Within-day maximum	124.2	300.4	241.0	901.4	15.0	18.1

Individual muscle fibre concentrations

A total of 207 individual muscle fibres (101 pre-supplementation; 106 post-supplementation) from the three horses were dissected, characterized and analysed for carnosine and taurine concentrations. Mean (± SD) carnosine and taurine concentrations in pre- and post-supplementation type I, IIA and IIB muscle fibres from the individual horses are given in Tables 7.2 and 7.3, respectively. Free histidine and ß-alanine were not detected in pre- or post-supplementation individual muscle fibres. The lower limits of quantitation for histidine and ß-alanine in individual muscle fibres by the method used were 1.0 and 0.4 mmol kg^{-1} DW. Mean carnosine and taurine concentrations in pre-supplementation type I, IIA and IIB individual muscle fibres from the three horses were in good agreement with values reported previously in Chapter 4. Following 30 days of histidine and ß-alanine supplementation the mean carnosine concentration increased in type IIA and IIB fibres in all three horses compared with pre-supplementation values. These increases reached statistical significance in three instances. In type IIA and IIB fibres from horse GT mean carnosine concentrations increased significantly by 31.2 and 43.8 mmol kg^{-1} DW ($p < 0.01$ and $p < 0.05$) or by 49% and 40% above the pre-supplementation contents, respectively. Mean carnosine concentration in type IIB fibres from horse JS also increased significantly by 22.1 mmol kg^{-1} DW ($p < 0.01$) or 17% above the pre-supplementation value. Owing to the presence of so few type I fibres it was difficult to make a realistic assessment of the significance of changes in carnosine concentration in type I muscle fibres. Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for the individual horses are shown in Figures 7.7 - 7.9.

There was no statistically significant difference in the taurine concentrations of type I, IIA and IIB before and after supplementation in any of the horses. However, for type I fibres the previously mentioned proviso applies.

Table 7.2 Mean (± SD) carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres from individual horses.

Horse	Treatment	Carnosine, mmol kg^{-1} DW (number of fibres)		
		Type I	Type IIA	Type IIB
DS	Pre	32.3 ± 14.5 (3)	72.1 ± 47.7 (11)	111.8 ± 22.8 (14)
	Post	-	76.2 ± 20.9 (17)	117.7 ± 38.7 (12)
JS	Pre	59.5 ± 3.9 (2)	102.6 ± 12.7 (12)	131.2 ± 26.6 (26)
	Post	55.5 (1)	112.2 ± 17.1 (18)	153.3 ± 28.0‡ (22)
GT	Pre	44.8 ± 6.6 (4)	63.6 ± 20.9 (10)	108.6 ± 41.5 (19)
	Post	37.0 ± 9.3 (2)	94.8 ± 30.2‡ (15)	152.4 ± 65.0† (19)

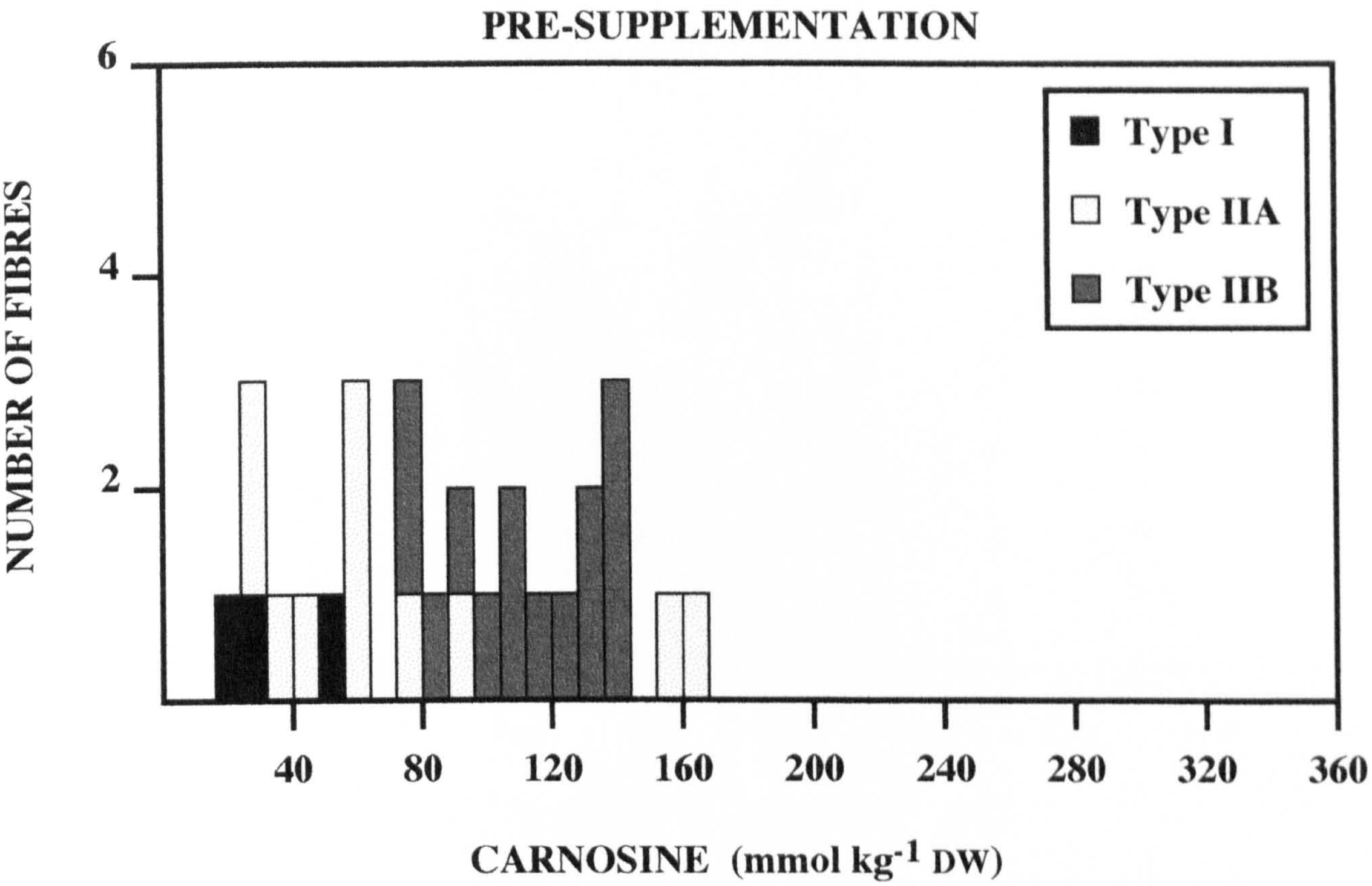
† = significantly different to pre, $p < 0.05$

‡ = significantly different to pre, $p < 0.01$

Table 7.3 Mean (± SD) taurine concentrations in pre- and post-supplementation type I, IIA and IIB fibres from individual horses.

Horse	Treatment	Taurine, mmol kg^{-1} DW (number of fibres)		
		Type I	Type IIA	Type IIB
DS	Pre	23.7 ± 4.2 (3)	3.1 ± 3.3 (11)	3.0 ± 2.1 (14)
	Post	-	1.8 ± 1.6 (17)	1.8 ± 3.2 (12)
JS	Pre	63.1 ± 16.1 (2)	1.7 ± 3.2 (12)	3.7 ± 4.6 (26)
	Post	54.0 (1)	3.4 ± 3.7 (18)	4.2 ± 3.1 (22)
GT	Pre	55.1 ± 52.7 (4)	5.8 ± 4.6 (10)	9.8 ± 8.3 (19)
	Post	23.0 ± 16.7 (2)	5.0 ± 5.8 (15)	7.1 ± 9.5 (19)

Figure 7.7 Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse DS.


PRE-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
Type I
Type IIA
Type IIB
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)

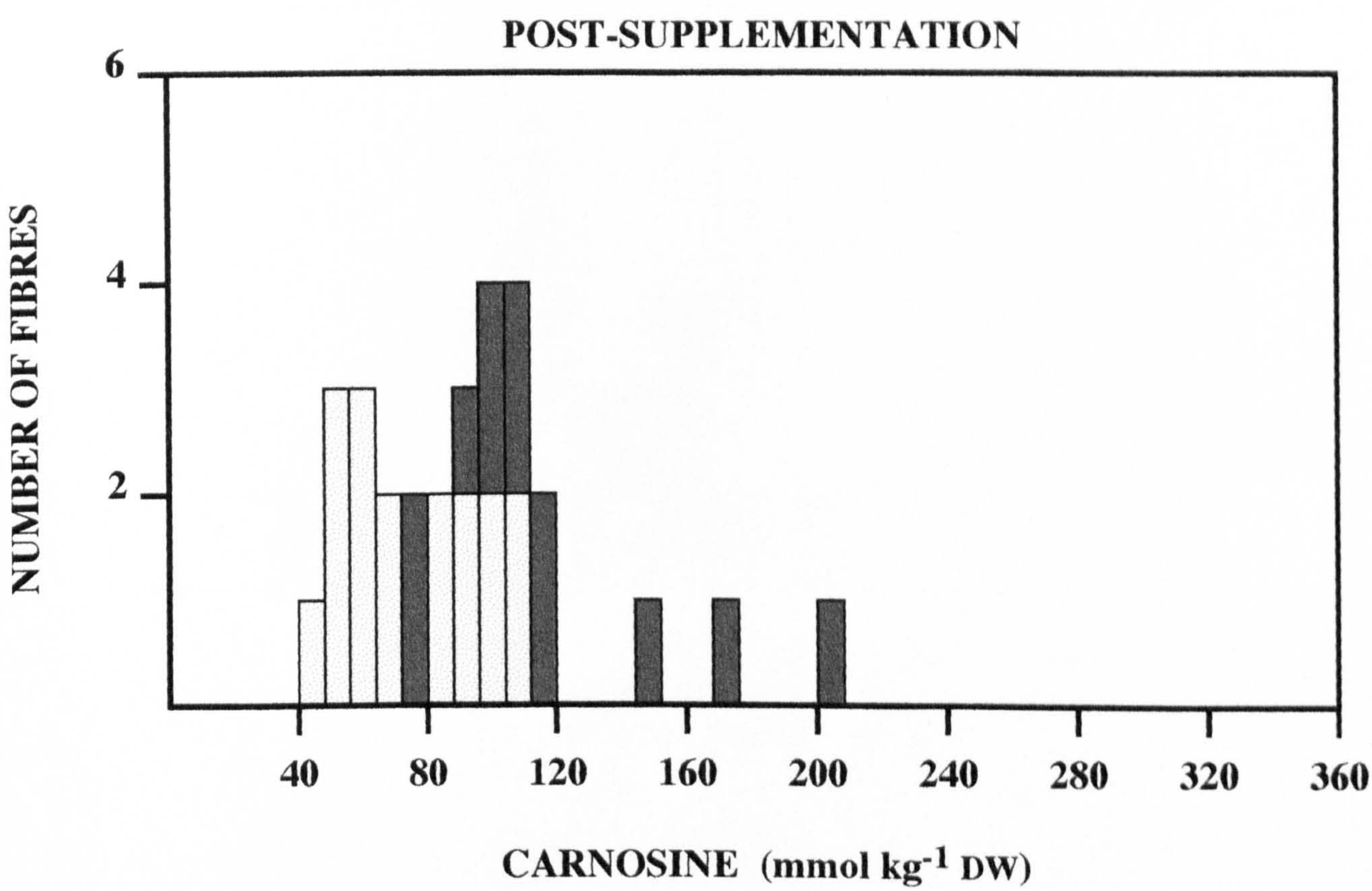
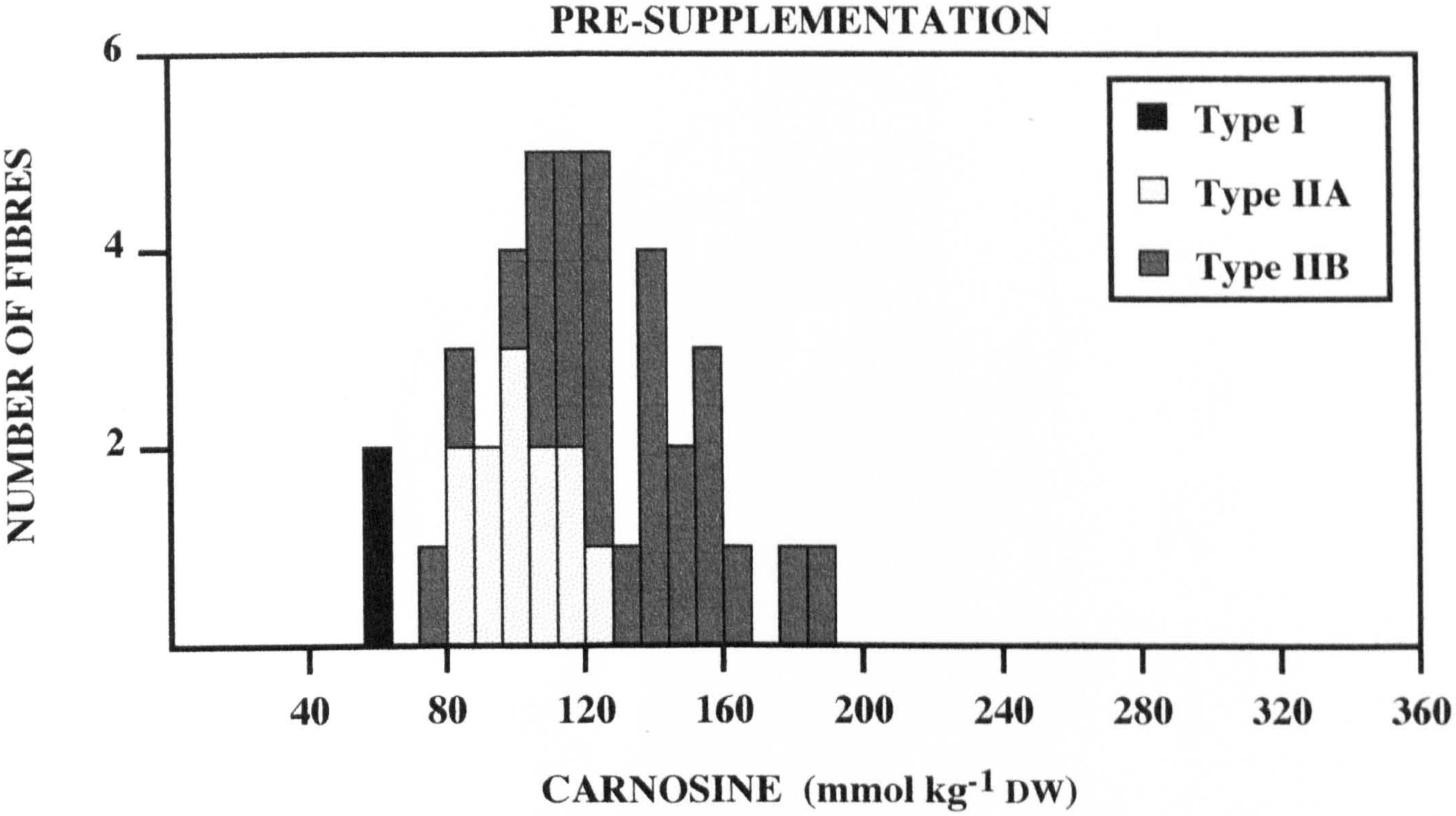

POST-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)

Figure 7.8 Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse JS.


PRE-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
Type I
Type IIA
Type IIB
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)

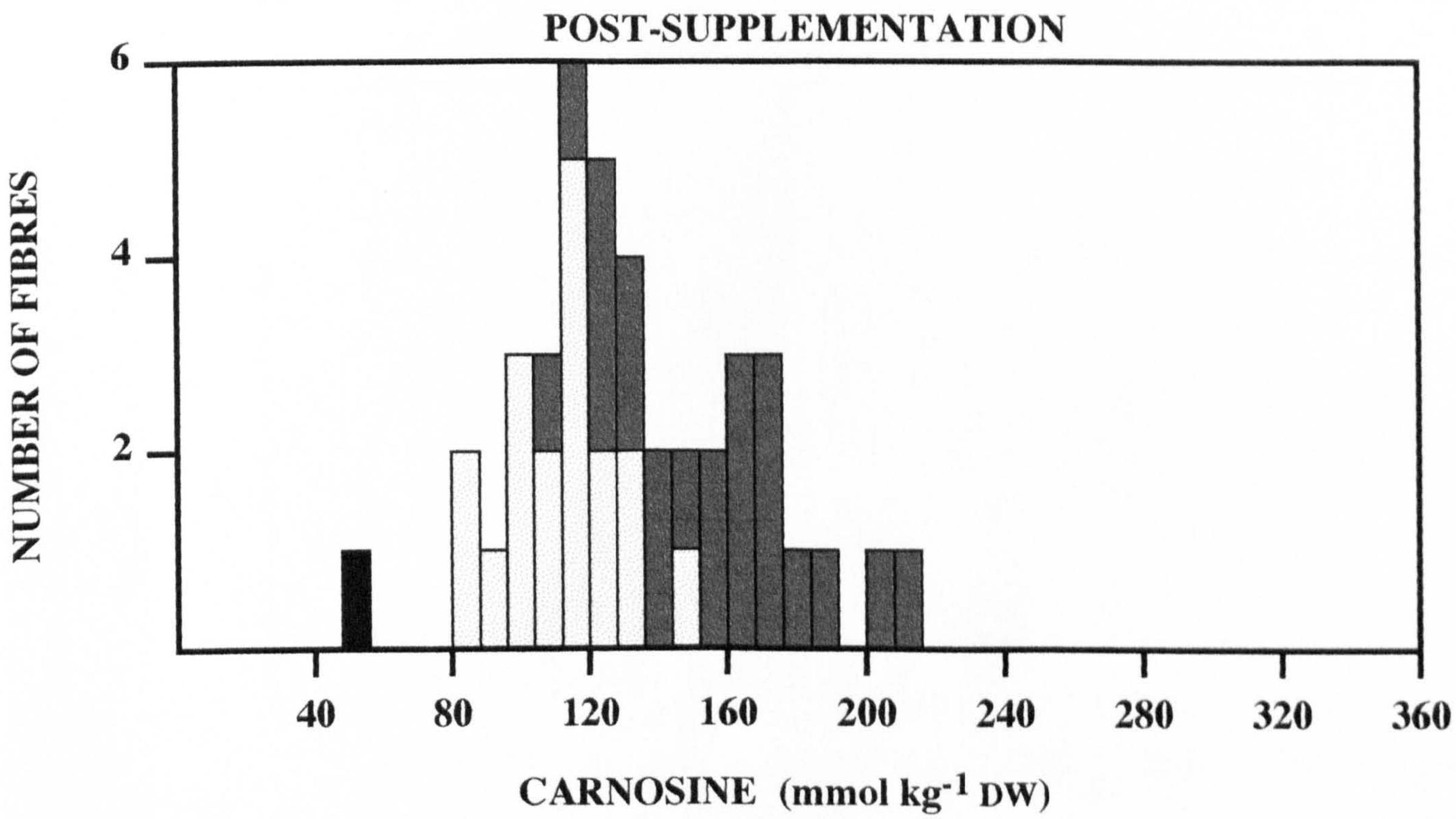
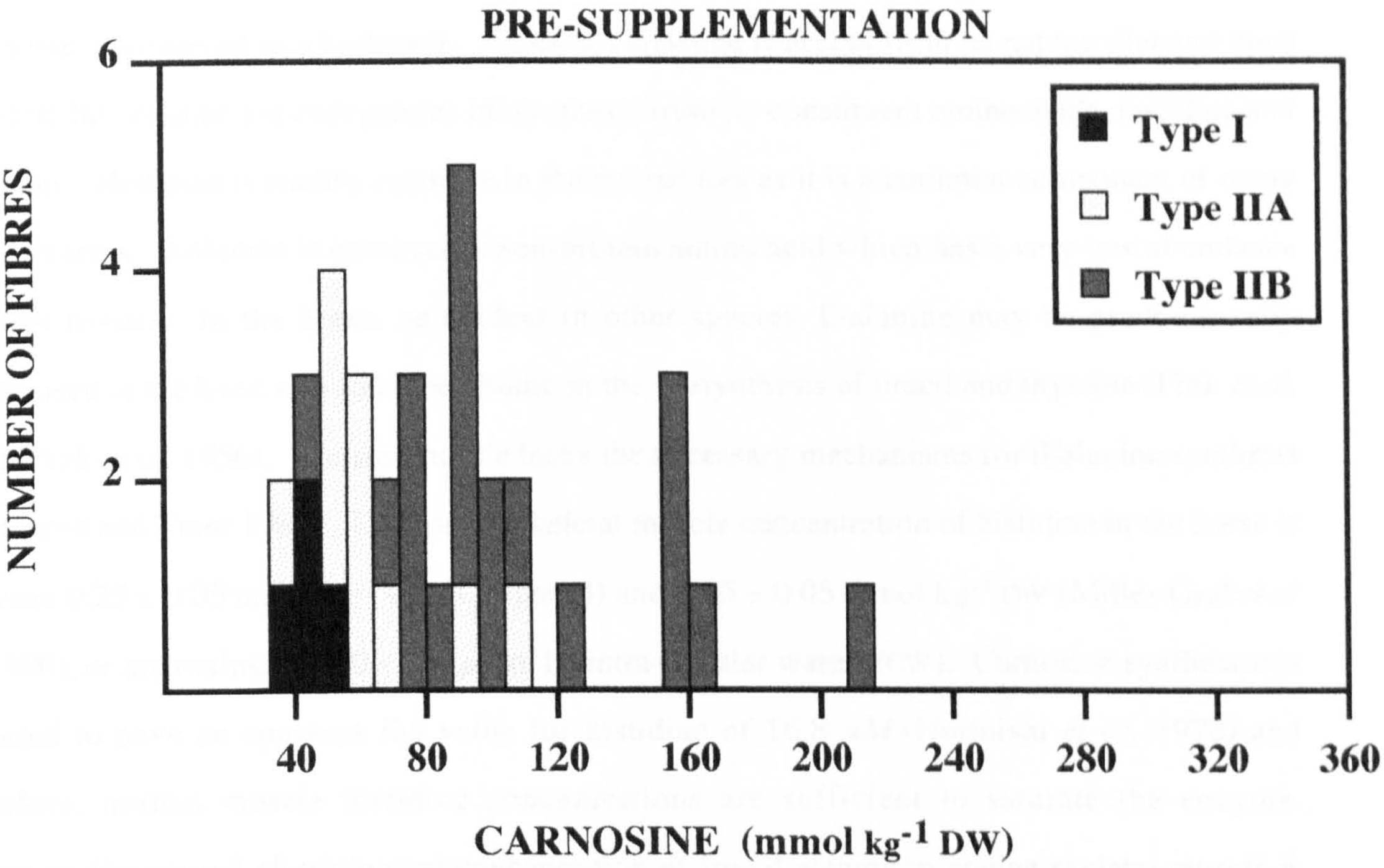
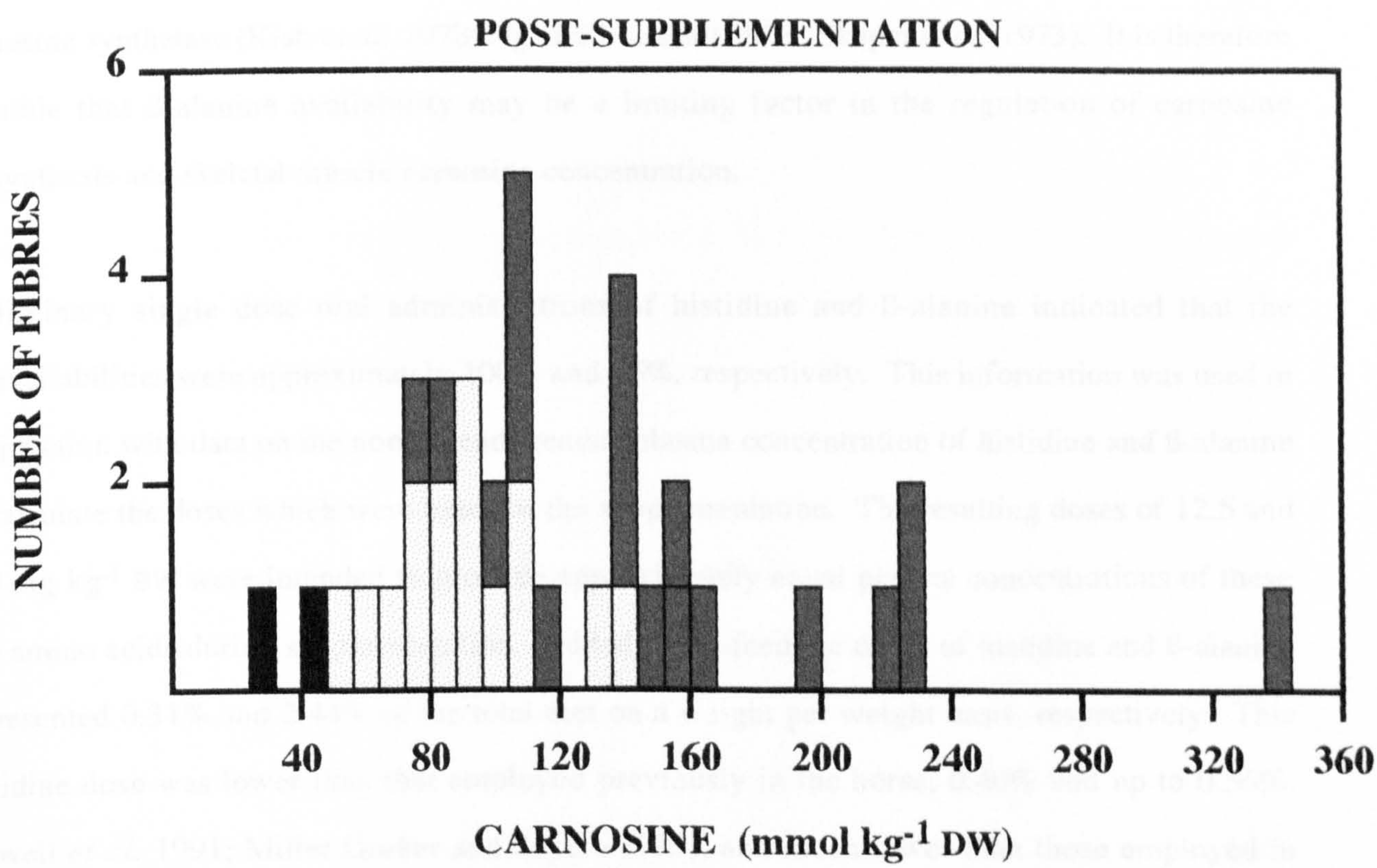

POST-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)

Figure 7.9 Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse GT.


PRE-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
Type I
Type IIA
Type IIB
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)


POST-SUPPLEMENTATION
NUMBER OF FIBRES
6
4
2
40
80
120
160
200
240
280
320
360
CARNOSINE (mmol kg-1 DW)

7.4 DISCUSSION

The horse has evolved as a herbivore. As such, carnosine is absent from its natural diet and must therefore be obtained via endogenous biosynthesis from its constituent amino acids, histidine and ß-alanine. Histidine is readily available in the equine diet as it is a common component of many plant proteins. ß-alanine is however, a non-protein amino acid which has a very low abundance in plant tissues. In the horse, as evident in other species, ß-alanine may be predominantly synthesised in the liver, as a final metabolite in the biosynthesis of uracil and thymine (Fink *et al.* 1953; Fink *et al.* 1956). Skeletal muscle lacks the necessary mechanisms for ß-alanine synthesis (Matthews and Traut 1987). The normal skeletal muscle concentration of histidine in the horse is between 0.25 ± 0.05 mmol kg^{-1} DW (Chapter 4) and 0.85 ± 0.05 mmol kg^{-1} DW (Miller-Graber *et al.* 1990), or approximately 80 - 280 μmol l^{-1} intra-cellular water (ICW). Carnosine synthetase is reported to have an apparent K_M value for histidine of 16.8 μM (Horinishi *et al.* 1978) and therefore, normal muscle histidine concentrations are sufficient to saturate the enzyme. However, the normal physiological concentration of free ß-alanine in equine skeletal muscle is 0.58 ± 0.17 mmol kg^{-1} DW or approximately 190 μmol l^{-1} ICW (Miller-Graber *et al.* 1990). This concentration is much lower than the K_M value of 1.0 - 2.3 mM ß-alanine previously reported for carnosine synthetase (Kish *et al.* 1978; Ng and Marshall 1978; Skaper *et al.* 1973). It is therefore possible that ß-alanine availability may be a limiting factor in the regulation of carnosine biosynthesis and skeletal muscle carnosine concentration.

Preliminary single dose oral administrations of histidine and ß-alanine indicated that the bioavailabilities were approximately 100% and 25%, respectively. This information was used in conjunction with data on the normal endogenous plasma concentration of histidine and ß-alanine to calculate the doses which were used for the supplementation. The resulting doses of 12.5 and 100 mg kg^{-1} BW were intended to produce approximately equal plasma concentrations of these two amino acids during supplementation. Added to the feed the doses of histidine and ß-alanine represented 0.31% and 2.44% of the total diet on a weight per weight basis, respectively. This histidine dose was lower than that employed previously in the horse, 0.40% and up to 0.56%, (Powell *et al.* 1991; Miller-Graber and Seyers 1993), and much lower than those employed in

other species, and the ß-alanine dose was twenty-fold lower that employed previously in mice (Margolis *et al.* 1985).

Plasma histidine concentrations on day 1 of the supplementation period were similar between horses DS and JS. The response in horse GT was somewhat lower. In contrast, horse GT displayed the greatest increase in plasma ß-alanine concentration during supplementation on day 1 than the other two horses. During the course of the 30 day supplementation period there was a progressive increase in both pre- and post-feeding histidine concentrations in all three horses and a convergence in the values between horses. By day 30 plasma histidine concentrations before and after feeding in horse GT were similar to the other horses. Post-feeding peak plasma histidine concentrations increased by up to 140%. Miller-Graber and Seyers (1993) reported an 80% increase in mean plasma histidine concentration in Quarterhorses during dietary histidine supplementation. A similar change in plasma histidine concentration has also been described in pigs during histidine supplementation (Izquierdo *et al.* 1988). Plasma ß-alanine concentrations showed much greater changes over the supplementation period. By day 30 ß-alanine was detectable in pre-feeding plasma and post-feeding peak plasma concentrations had increased by 300 - 400%. These changes in plasma histidine and ß-alanine concentrations throughout the supplementation period indicate an adaptive response to the prolonged administration of these amino acids which is more pronounced for ß-alanine than for histidine. Differences in the magnitude of the adaptive responses for histidine and ß-alanine probably arose as a consequence of the existence of different membrane transport mechanisms for α-amino acids and ß-amino acids. Distinct highly specific but low capacity transport mechanisms for the ß-amino acids, ß-alanine, taurine and γ-aminobutyric acid, have been demonstrated in the GIT of several species (Munck and Munck 1992; Munck and Munck 1994; Navab *et al.* 1984) and in chick pectoral muscle cells in primary culture (Bakardjiev and Bauer 1994).

Increases in the carnosine concentrations in type I, IIA and IIB fibres as a result of histidine and ß-alanine supplementation differed markedly between horses and on visual inspection of the data appeared to be correlated to adaptive increases in plasma ß-alanine concentration over the 30 day

supplementation period rather than to changes in plasma histidine concentration. This is consistent with the results of Powell *et al.* (1991) and Miller-Graber and Seyers (1993) where despite larger histidine doses no significant increase in middle gluteal muscle carnosine concentration was detected during histidine supplementation alone. The greatest increase, which was evident in both type IIA and IIB fibres, was found in horse GT. This correlates with the greater adaptive response to ß-alanine supplementation found in this horse as shown by changes in plasma ß-alanine concentration. Horse JS also showed a significant increase in the carnosine concentration in type IIB fibres which correlates with the lesser though sustained increase in plasma ß-alanine concentration. No significant change in the carnosine concentrations in type IIA and IIB fibres in horse DS is supported by the fact that a significant increase in plasma ß-alanine concentration was only recorded on day 30, which suggests a slower adaptation to ß-alanine supplementation.

The increase in the carnosine content of equine skeletal muscle, resulting from increased biosynthesis during dietary supplementation would therefore result in a corresponding increase in the total intra-cellular physico-chemical buffering capacity of the muscle ($ßm_{total}$). An increase in $ßm_{total}$ arising from an increase in the muscle carnosine concentration ($ßm_{carnosine}$) can be determined by using the Henderson-Hasselbach equation to calculate the increase in the protonated from of carnosine (pK_a 6.83) over the pH range 7.1 - 6.5, which represents the typical change in intra-cellular pH during high-intensity (fatiguing) exercise:

$$pH = pK_a + \log_{10}([Salt]/[Acid])$$

Hence, for a muscle carnosine concentration of 100 mmol kg^{-1} DW the quantities of carnosine in the protonated form at pH 7.1 and 6.5, respectively are:

$$pH\ 7.1\ [Acid] = 100/[1 + \text{antilog}_{10}(7.1 - 6.83)]$$

$$= 34.9 \text{ mmol kg}^{-1} \text{ DW}$$

$$pH\ 6.5\ [Acid] = 100/[1 + \text{antilog}_{10}(6.5 - 6.83)]$$

$$= 68.1 \text{ mmol kg}^{-1} \text{ DW}$$

$$\Delta \text{ [Acid] pH 7.1 - 6.5} = 33.2 \text{ mmol } H^+ \text{ kg}^{-1} \text{ DW}$$

The increase in the protonated from of carnosine represents the amount of H^+ ions buffered by an intra-muscular carnosine concentration of 100 mmol kg^{-1} DW. For example, in horse GT the mean carnosine concentrations in the type IIB fibres before and after supplementation were 108 and 152 mmol kg^{-1} DW, respectively.

For the pre-supplementation type IIB fibre carnosine concentration of 108 mmol kg^{-1} DW:

$$\Delta \text{ [Acid]} = \{108/[1 + \text{antilog10 } (6.5 - 6.83)]\} - \{108/[1 + \text{antilog10 } (7.1 - 6.83)]\}$$

$$ß m_{\text{carnosine}} = 35.9 \text{ mmol } H^+ \text{ kg}^{-1} \text{ DW}$$

For the post-supplementation type IIB fibre carnosine concentration of 152 mmol kg^{-1} DW:

$$\Delta \text{ [Acid]} = \{152/[1 + \text{antilog10 } (6.5 - 6.83)]\} - \{152/[1 + \text{antilog10 } (7.1 - 6.83)]\}$$

$$ß m_{\text{carnosine}} = 50.5 \text{ mmol } H^+ \text{ kg}^{-1} \text{ DW}$$

The increase in the mean carnosine concentration in type IIB fibres as a result of supplementation would produce an increase in $ß m_{\text{carnosine}}$ of 14.6 mmol H^+ kg^{-1} DW or 41%. The same calculation performed using the carnosine concentration in type IIA fibres from horse GT before and after supplementation gives an increase in $ß m_{\text{carnosine}}$ of 10.4 mmol H^+ kg^{-1} DW, or 49% as a result of supplementation.

Titremetric measurements previously used have estimated the total intra-cellular physico-chemical buffering capacity of skeletal muscle ($ß m_{\text{total}}$) in the thoroughbred horse to be 117 ± 8.5 mmol H^+ kg^{-1} DW over the pH range 7.1 - 6.5 (Harris *et al.* 1990). Subtraction of the buffering capacity directly attributable to carnosine ($ß m_{\text{carnosine}}$), as calculated from the carnosine concentration using the Henderson-Hasselbach equation, indicated the residual buffering capacity from the effects of protein and phosphates ($ß m_{\text{residual}}$) to be 81.8 ± 9.7 mmol

H^+ kg^{-1} DW (Harris *et al.* 1990). The use of multiple linear regression analysis enabled estimates to be made of the residual buffering capacity to be made in type I, IIA and IIB fibres. $ßm_{residual}$ was shown to be relatively constant in the three fibre types with a value between 68 and 72 mmol H^+ kg^{-1} DW (Sewell *et al.* 1990). Assuming a mean value of 70 mmol H^+ kg^{-1} DW for residual buffering capacity in type IIB fibres it can be calculated from the example of the horse (GT) above that $ßm_{total}$ in type IIB fibres as a result of supplementation would have increased by 14%. The same approach indicates that $ßm_{total}$ in type IIA fibres from this horse as a result of supplementation would have increased by 11%. Estimated values of $ßm_{total}$ and $ßm_{carnosine}$ before and after supplementation and the increase in $ßm_{total}$ (%) in type I, IIA and IIB fibres for the individual horses are given in Table 7.4.

During high-intensity exercise, such as that encountered during Thoroughbred flat-racing, a high rate of ATP production is maintained through anaerobic glycolysis. However, this incurs a rapid production and accumulation of lactic acid within the skeletal muscles. Dissociation of the lactic acid into lactate ions and H^+ ions results in a progressive increase in H^+ ion concentration and subsequent fall in intra-cellular pH. In turn, this reduction in intra-cellular pH can impair the integrity of the various mechanisms involved in muscle contraction, such as ATP resynthesis, which may result in localized muscle fatigue. An increase in the intra-cellular physico-chemical buffering capacity of equine skeletal muscle, through an increase in skeletal muscle carnosine concentration, should attenuate the rate of decline in intra-cellular pH during high-intensity exercise thus enabling the maximal speed attained to be sustained for a longer period prior to the onset of fatigue.

Table 7.4 Estimates of βm_{total} and $\beta m_{carnosine}$ before and after supplementation and the increase in βm_{total} (%) in type I, IIA and IIB fibres for the individual horses.

Fibre type	Buffering capacity	DS		JS		GT	
		Pre	Post	Pre	Post	Pre	Post
Type I	$ßm_{carnosine}$	10.7	-	19.8	18.4	14.9	12.3
	$ßm_{total}$	80.7	-	89.8	88.4	84.9	82.3
	$\Delta ßm_{total}$ (%)				- 1.6		- 3.1
Type IIA	$ßm_{carnosine}$	23.9	25.3	34.1	37.2	21.1	31.5
	$ßm_{total}$	93.9	95.3	104.1	107.2	91.1	101.5
	$\Delta ßm_{total}$ (%)		1.5		3.0		11.4
Type IIB	$ßm_{carnosine}$	37.1	39.1	43.6	50.9	36.1	50.6
	$ßm_{total}$	107.1	109.1	113.6	120.9	106.1	120.6
	$\Delta ßm_{total}$ (%)		1.9		6.4		13.7

CHAPTER 8

GENERAL DISCUSSION

The imidazole dipeptide carnosine (ß-alanyl-L-histidine) and its methylated derivatives anserine (ß-alanyl-L-1-methylhistidine) and balenine (ß-alanyl-L-3-methylhistidine), are found predominantly in vertebrates and occur almost exclusively in the skeletal muscles (Crush 1970; Suyama *et al.* 1970; Carnegie *et al.* 1983; Plowman and Close 1988). Although they can all be found in varying proportions in a given species carnosine is usually dominant in terrestrial mammals, anserine in birds and balenine in aquatic mammals and reptiles. The carnosine concentration in the skeletal muscle of the horse is the highest found in any terrestrial mammal. In the eighty or so other species of animals studied only a very few such as the Tuna, sea snakes and some deep-diving whales have greater skeletal muscle imidazole dipeptide contents, predominantly balenine (Crush 1970; Suyama *et al.* 1970). The typical carnosine concentration in equine skeletal muscle is generally in excess of 100 mmol kg^{-1} DW, a concentration which easily exceeds that exhibited by other small molecules including phosphocreatine, creatine, ATP and taurine. This probably makes carnosine the most abundant low molecular mass compound present in mammalian skeletal muscle. The occurrence of such high intra-muscular carnosine concentrations in the horse is inextricably linked to its evolutionary adaptation to high speed running wherein muscle contraction is reliant upon anaerobic glycolysis to provide a rapid turnover of ATP. As a consequence of this energy producing pathway, there is a high rate of production and subsequent accumulation of lactic acid which dissociates into lactate and H^+ ions within the working muscles. Muscle lactate concentrations as high as 200 mmol kg^{-1} DW have been observed in the horse following racing (Valberg 1987) and experimental exercise studies (Snow *et al.* 1985). In the absence of an effective mechanism to counter this large accumulation of H^+ ions (acidosis) the intra-muscular pH would theoretically fall to a value of approximately pH 1.0; a value well below that which is physiologically tolerable. In practice, however, during high-intensity exercise intra-cellular pH can decline from a normal resting level of pH 7.1 to a post-exercise value of pH 6.5 or slightly less (Harris *et al.* 1987). Even such a relatively high post-exercise pH can impair the contractile process and result in localized muscle fatigue and loss of performance. Therefore the maintenance of intra-muscular pH homeostasis may be as important to athletic performance as the ability to transport oxygen via the cardio-vascular system.

The horse, like many species, has evolved a system for the intra-cellular physico-chemical buffering of H^+ ions in skeletal muscle comprising several different components, such as protein histidine residues, inorganic and organic phosphates, and the imidazole dipeptides including carnosine, which act in concert to provide the overall buffering capacity. Carnosine has a pK_a value of 6.83 (Tanokura *et al.* 1976) which enables it to function as an effective H^+ ion buffer over the physiological pH range and it has been calculated that carnosine accounts for approximately 30% of the total intra-cellular physico-chemical buffering capacity in equine skeletal muscle (Sewell *et al.* 1991). The concentrations in skeletal muscle of the aforementioned buffering constituents, protein histidine residues, organic and inorganic phosphate are constrained within narrow physiological limits which allows limited scope for an increase in their muscle content in response to evolutionary demands for greater H^+ buffering capacity. Much greater flexibility exists for increases to the imidazole dipeptide concentration in skeletal muscle in order to enhance total buffering. This adaptive response to the need for greater buffering is exemplified by differences between three athletic species, namely horses, dogs and humans. The total physico-chemical buffering capacity is higher in horses than in dogs and lowest in humans (Harris *et al.* 1990). However, the residual buffering capacity attributable to the histidine residues in the muscle structural proteins actin and myosin, and also phosphates is almost identical in each species. The differences between total buffering and residual buffering between species is attributable to the different concentrations of the imidazole dipeptides (Harris *et al.* 1990). This potential is visibly exploited in equine skeletal muscle where its high anaerobic, capacity and therefore considerable capacity for H^+ ion production, is compensated for by an equally large carnosine concentration.

There is considerable experimental evidence which clearly demonstrates the importance of carnosine towards intra-cellular physico-chemical H^+ ion buffering capacity. The middle gluteal muscle is a principal locomotory muscle in the horse. The mean carnosine concentration in this muscle (107.8 mmol kg^{-1} DW), as determined in Chapter 4, is in agreement with previously established values (Marlin *et al.* 1989; Harris *et al.* 1990), but is significantly higher than concentrations found in 'non-locomotory' skeletal muscles, such as the internal intercostal (63.7

mmol kg^{-1} DW), and in the diaphragm (38.6 mmol kg^{-1} DW) both of which can be considered to be 'non-skeletal' muscle. These latter two muscles have no direct role in the mechanics of locomotion, in contrast to the middle gluteal. As such they are likely to have a low glycolytic capacity and low potential for H^+ ion production, and consequently a lower buffering requirement. The same argument can be used to explain the very low carnosine concentrations found in cardiac muscle (2.2 mmol kg^{-1} DW) and the smooth muscle of the GIT (0.6 - 2.4 mmol kg^{-1} DW) in the horse. Furthermore, carnosine concentrations in non-muscle tissues including the liver and kidney are approximately twenty-fold lower than the cardiac muscle values.

Comparative measurements of carnosine concentrations and the relative proportions of type I, IIA and IIB fibres present, in muscle samples from different regions of the middle gluteal muscle, indicated that the carnosine content was positively correlated to type II % FSA and negatively correlated with type I % FSA (Sewell *et al.* 1991). From this it was estimated that the carnosine concentrations in type I, IIA and IIB fibres from horses of various ages and states of training were 21, 86 and 116 mmol kg^{-1} DW, respectively (Sewell *et al.* 1991). The development of the analytical methodology described in Chapter 3 (Dunnett and Harris 1995b) enabled the direct measurement of carnosine concentrations in individual type I, IIA and IIB muscle fibres from the middle gluteal of the thoroughbred horse, as described in Chapter 4. The measured carnosine concentrations in type I, IIA and IIB fibres were 21, 95 and 104 mmol kg^{-1} DW, respectively (Dunnett and Harris 1995a). The higher concentration in type II fibres in contrast to type I fibres is consistent with the physiological and metabolic differences between them (Snow 1983) and the recruitment of type II fibres during high-intensity exercise (Lindholm *et al.* 1974; Snow *et al.* 1982). This provides further evidence for the importance of carnosine to H^+ buffering in the horse. The lower concentration of carnosine found in type I fibres is, however, relatively high in comparison with other muscle metabolites, and may be explained partly through the necessity to buffer H^+ ions diffusing from adjacent type II fibres, and partly through its proposed antioxidant function which provides protection to membrane lipids against damage from reactive oxygen species, such as $OH^{\bullet}$ radicals, HOCl, H_2O_2 and singlet oxygen, (Boldyrev *et al.* 1988; Kohen *et al.* 1988) which can be generated in this highly oxidative tissue.

Higher muscle carnosine contents have been observed in horses selectively bred for higher intensity exercise. American Quarterhorses have mean middle gluteal muscle carnosine concentrations of approximately 159 mmol kg^{-1} DW, in comparison to values of 125 mmol kg^{-1} DW in Thoroughbreds and 111 mmol kg^{-1} DW in Standardbreds (Bump *et al.* 1990). Although carnosine undoubtedly makes an important contribution to total intra-cellular H^+ ion buffering in skeletal muscle and, therefore in all probability, to the capacity for sprint exercise in the horse, there has been no previous direct experimental evidence demonstrating an increase in muscle carnosine concentration as a consequence of training. However, estimates of the carnosine contents in the different muscle fibre types were generally higher in exclusively highly trained thoroughbred horses in contrast to horses of mixed training (Sewell *et al.* 1992). Data from Chapter 4 suggests that long-term intensive training with a significant sprint exercise component can result in higher carnosine concentrations in all three fibre types in the middle gluteal muscle of young thoroughbred horses in contrast to non-trained horses of the same age. Although the difference in carnosine concentration in the type I and type IIB fibres between the trained and untrained groups did not reach statistical significance, a large increase was observed in the carnosine content of the type IIA fibres. The mean carnosine concentration in type IIA fibres from the trained horses was 92 mmol kg^{-1} DW in contrast to a value of only 68 mmol kg^{-1} DW in the untrained horses. It is possible that this increase in the type IIA fibres rather than the type IIB fibres reflects a greater 'trainability' of the oxidative-glycolytic type IIA fibres in the horse.

Carnosine is present in the plasma of the normal resting thoroughbred horse at concentrations ranging from approximately 5 to 20 μM. Furthermore, the plasma concentration in a given horse is relatively constant during a 24 h period and is unaffected by fasting. The constancy of the plasma carnosine concentration may in part be due to the minimal influence of the diet. The horse is an herbivorous animal and as such carnosine is not a normal constituent of the diet. The mean within-horse variance in plasma carnosine concentration ($\pm$ 1.6 μM) over of the second half of a 24 h period during a triple feed regime was almost identical to that found during the same period during a 24 h fast ($\pm$ 1.5 μM). Other factors however, such as age, exercise and muscle damage may exert a variable influence on the plasma carnosine concentration both in the

short and the long term. In foals and yearlings, the concentration of carnosine in the plasma is significantly lower than that found in older horses. In other mammals carnosine is synthesized almost exclusively in skeletal muscle. As the animal matures the muscle carnosine content, increases (Christman 1976; Johnson and Hammer 1992), probably due to an increased biosynthetic capacity. Therefore, the age dependent increase in equine plasma carnosine concentration most probably arises from increased 'leakage' of carnosine from skeletal muscle associated with a higher rate of carnosine turnover in mature horses. Furthermore, plasma carnosinase activity present in humans and other primates is completely absent in equine plasma. Short duration high-intensity exercise can also cause a small, up to two-fold, elevation in plasma carnosine concentration, possibly due to minor muscle damage. Still larger increases in circulating levels of carnosine of as much as 700 μM , can occur during episodes of ERS and probably arise from the occurrence of extensive muscle damage or changes in sarcolemmal permeability. This is suggested also by correspondingly large increases in the plasma activities of the muscle enzymes AST and CK.

Carnosine administration to the horse via the intra-venous route produces a large but short term increase in the plasma carnosine concentration. Pharmacokinetic calculations indicate that the elimination half-life of carnosine from the central compartment, which includes the systemic circulation, is approximately 2 h, and that plasma carnosine concentrations have almost returned to normal within 8 h. This data in conjunction with a low value for the apparent volume of distribution at steady-state suggests that carnosine uptake into the peripheral compartment, which includes the skeletal muscle, is minimal. Although urinary excretion of unmetabolized carnosine can account for as much as 36% of the administered dose following intra-venous injection, there is a large between-horse variance in the proportion of the dose eliminated from the body in this manner. The fate of the remainder of the administered carnosine dose can probably be accounted for through carnosinase and non-specific dipeptidase catalysed degradation to histidine, with subsequent re-uptake of the amino acid by various tissues as evinced by a delayed and sustained increase in the plasma histidine concentration observed after intra-venous carnosine administration and the absence of increased urinary histidine excretion.

Orally administered carnosine is absorbed from the GIT of the thoroughbred horse and subsequently produces an increase in the plasma carnosine concentration. A large part of the dose is metabolized to histidine at an initial rate similar to the rate of absorption of carnosine resulting in an increase in the plasma histidine concentration. The coincidence of the peak plasma histidine and carnosine concentrations suggests that the majority of the carnosine hydrolysis occurs within the GIT, although a first-pass effect in the liver may also contribute. The greater carnosinase and non-specific dipeptidase activity observed in the equine GIT compared with the liver supports this. The proportion of the absorbed carnosine dose which appears as histidine in the plasma decreases as the size of the dose is increased suggesting that the enzymatic hydrolysis is becoming saturated. The bioavailability of carnosine via the oral route increases proportionally with the size of the dose. However, the bioavailability is very low with only 4% of the total administered dose entering the systemic circulation at the highest dose used. The low bioavailability of carnosine from the GIT is probably due in part to its extremely lipophobic nature which prevents diffusion across the membranes, and to the absence or low capacity of a specific membrane transporter. Owing to its low bioavailability, oral carnosine supplementation as a means to enhance the endogenous muscle concentration in the thoroughbred horse would be ineffective. Furthermore, the low $V_{d(ss)}$ of 0.22 l kg^{-1} and short $t_{1/2(\beta)}$ of 162 min suggest that carnosine does not penetrate extensively into the intra-cellular space of low blood-flow tissues, such as skeletal muscle.

Dietary supplementation with the amino acid precursors of carnosine, histidine and ß-alanine, has the potential to increase the endogenous muscle carnosine concentration. Studies in other mammalian species have indicated that carnosine biosynthesis occurs within the skeletal muscles. However, the endogenous ß-alanine concentration of 0.58 mmol kg^{-1} DW in equine skeletal muscle (Miller-Graber *et al.* 1990) is lower than its reported K_M for carnosine synthetase (Skaper *et al.* 1973; Kish *et al.* 1978; Ng and Marshall 1978), and therefore ß-alanine availability may be a limiting factor to carnosine biosynthesis *in vivo* . In Chapter 7, sustained dietary supplementation with histidine and ß-alanine in the thoroughbred horse produced increases in skeletal muscle carnosine concentrations in all horses. However, there was considerable

variability in the extent of this increase both between horses and between the different fibre types. Statistically significant increases were observed in only two of the three horses. Both of these demonstrated a significant increase in the type IIB fibre carnosine concentration, whereas only one showed a significant increase in the type IIA fibre carnosine concentration. It was not possible to assess the effect of supplementation on the carnosine concentration in type I fibres owing to the small number of these fibres obtained from the biopsy samples.

In general, sustained ß-alanine supplementation resulted in a progressive day-to-day increase in plasma ß-alanine concentration, although this parameter also showed a large between-horse variability. This progressive response to continued ß-alanine supplementation was probably due to an adaptive increase in the gastro-intestinal bioavailability of ß-alanine. Changes in plasma histidine concentrations over the same period were much less marked. The extent of the increase in skeletal muscle carnosine content in a given individual appeared to be related to the magnitude of the adaptive increase in plasma ß-alanine concentration within that individual. This supports the earlier hypothesis that ß-alanine availability is a limiting factor to *in vivo* carnosine biosynthesis.

The higher carnosine concentrations present in type IIA and IIB fibres in contrast to type I fibres suggests a greater capacity for carnosine biosynthesis in the former. This could be established by an investigation of the distribution of carnosine synthetase activity in the different fibre types. Following the observation of an increase in the carnosine concentration in type IIA fibres with training, it would be of interest to determine whether this was accompanied by an adaptive increase in the activity of carnosine synthetase in these fibres, and whether previously observed increases in muscle buffering capacity during high-intensity training (McCutcheon *et al.* 1987; Sinha *et al.* 1991) can be accounted for by increases in the muscle carnosine content. Further work could investigate whether an increased skeletal muscle carnosine concentration, following long-term dietary supplementation with ß-alanine and histidine, influences the metabolic responses to maximal exercise, such as a delayed onset of metabolic acidosis, and a reduction in

muscle ATP depletion and IMP accumulation at the single fibre level, and plasma ammonia production.

Several practical applications of the information gained from the current research may be envisaged. The release of carnosine into the systemic circulation can occur under various conditions, such as during training and racing, episodes of ERS, or as a result of trauma, and is indicative of muscle damage. The principal advantage of using equine plasma carnosine concentration as a clinical indicator of skeletal muscle damage is that owing to the extremely low carnosine concentrations in other tissues it is essentially a specific indicator of skeletal muscle damage. This is in contrast to AST and CK. If sufficient serial blood samples were collected the total amount of carnosine released and hence the mass of tissue affected could be estimated from comparison of the AUC data with that calculated from a known intra-venous bolus dose, or more accurately from an intra-muscular bolus injection.

It has been reported that during episodes of ERS there appears to be selective damage to type II skeletal muscle fibres (Valberg *et al.* 1993). Then on this basis, since the distribution of carnosine is compartmentalized mostly into type IIA and IIB fibres, and taurine into type I fibres, during an episode of ERS a significant increase in plasma carnosine concentration only should be observed. However, this is contradicted by the plasma data in Chapter 4 from two of the horses which were presented with episodes of ERS. A significant increase in plasma taurine concentration was also observed in each case. Because of their heterogeneous distribution increases in the plasma concentration of carnosine or taurine may in theory be used as indicators of selective type II or type I muscle fibre damage, respectively.

The compartmentalization of carnosine into type II fibres and taurine into type I fibres may enable them to be used as physico-chemical markers of the respective fibre types. This could be exploited by nuclear magnetic resonance imaging techniques to map the regional distribution of type I and type II fibres in the skeletal muscles and the relative proportions of each fibre type in the whole tissue. In principal it would be possible to extrapolate the technique to estimate these

relative proportions in all the locomotory muscles, and therefore to make an assessment of the athletic potential of an individual for endurance or sprint type exercise.

Experimentally, sustained oral administration of ß-alanine and histidine appears to be an effective method for increasing the endogenous skeletal muscle carnosine concentration in the thoroughbred horse. An increased skeletal muscle carnosine concentration would result in a greater H^+ ion buffering capacity which is likely to reduce the rate of metabolic acidosis during high-intensity exercise and subsequently may delay the onset of local muscle fatigue. It is therefore possible that a dietary supplement comprising these two amino acids could be used as an ergogenic aid to enhanced performance during sprint exercise.

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